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Enhancements of Cellular Therapy for Ischemic Stroke

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Enhancements of Cellular Therapy for Ischemic Stroke

By

Todd C. Deveau
B.S., Vanderbilt University, 2005

Advisor: Ling Wei, M.D.

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 Sciences
Neuroscience
2015

Abstract

Enhancements of Cellular Therapy for Ischemic Stroke
By Todd C. Deveau

Ischemic stroke is a leading cause of death and disability in the United States. Treatment options are currently limited, and currently only one FDA-approved therapy exists (tPA). Although tPA was a great stride forward in stroke treatment, only a small percentage of patients are eligible to receive the drug and even smaller actually receive it. Research to date has focused on small molecule and peptide based therapies aimed at neuroprotection: protecting dying neurons, glia, and vascular from ischemia-media cell death. Decades of pre-clinical studies generated exciting results in animal models, however all have failed to translate to successful patient care. Currently, there are no therapies available to promote central nervous system (CNS recovery) in stroke survivors. Cellular therapy, the use of stem cells for stroke treatment, has blossomed in the lab in the past decade. Induced pluripotent stem cells (iPS cells, or iPSCs) in particular are an excellent candidate for future stroke therapies. With multiple mechanisms of action and the possibility for not only neuronal cell replacement but personalized medicine as well, the use of iPSCs for stroke therapy holds great potential. Current studies generate as proof-of-principal that iPSC transplantation can be safe, efficacious, and can provide a source of new neurons that integrate into host circuitry. While these studies are exciting, they are far from ideal. Many questions remain and in its current form, iPSC transplantation for stroke is not optimized and remains inefficient. This dissertation examines how iPSC transplantation may be optimized for ischemic stroke treatment. We examined a novel intranasal transplantation route as well as a novel genetic manipulation method (focal adhesion kinase, FAK, overexpression we hypothesized would improve functional recovery over current methods. Our results suggest that the novel non-invasive intranasal transplantation may improve functional recovery at a similar level compared to existing methods. We also demonstrate FAK overexpression may improve some aspects of normal neuronal development that would aid the integration of transplantated cells. Taken together, our data suggest that optimization of existing iPSC transplantation methods may benefit iPSC therapy for ischemic stroke.

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Acknowledgments

The chapters that follow describe years of hard work done through hard work and more importantly, teamwork. First and foremost, Dr. Ling Wei has provided me with the utmost professional and personal support to persevere through the years of my study. Her best interests were always mine and her own dedication and commitment to her career will serve as a model for my own. Special thanks to my committee, Dr. Shan Ping Yu, Dr. Peng Jin, Dr. Thaddeus Pace, and Dr. Wen-Cheng Xiong, as their guidance and enthusiasm over many years helped me to persevere through the current study. Within the lab (in no particular order), Dongdong Chen, Xiaohuan (Hannah) Gu, Dr. Mingke (Mike) Song, Jinmei Sun, Ning Wei, Lili Wang, Yingying Zhao, Dr. Jinhwan Lee, Dr. Zheng (Zach) Wei, Dr. Wenyuan (Cody) Cao, Michael Jiang, and James Zhang were dedicated lab partners, reliable hands, solid sounding boards, and friends. Special thanks to Michael Jiang and Katharine Yuengling, who assisted with the behavioral assays and immunostaining in Chapter IX. The bulk of this work was also done with guidance and in partnership with fellow graduate students Molly Ogle, Osama Mohamad, and Monica Chau. I'm very thankful for their help, support, and friendship without which this body of work would not have been possible. Thanks additionally to the Emory Viral Vector Core and the Cloning Core. Emory provided a very supportive research environment, and facilities such as those two core facilities allowed my work to progress. Many thanks to my classmates for the nurturing environment that is Emory Neuroscience; I am blessed to have been able to find life-long friends during this journey and for their friendship I am grateful. I am very thankful for the support provided by my family as well; my parents and brother Patrick for supporting me, pushing me to succeed, and setting the bar for success high. I am thankful for my grandparents and the pride they have taken in me and the work I have done. Even though not everyone saw me finish the journey, I know everyone is still with me in some way. Last, big thanks to my Hannah, who I'm very fortunate to have in my life for love, humor and support, especially these few months as I've tried to finish my studies.

Of course, a special recognition of the rodents who help to advance our understanding of health and disease every day.

List of Figures

List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
Brdu	5-bromo-2'-deoxyuridine
CCA	common carotid artery
CNS	central nervous system
DIV	days in vitro
DCX	doublecortin
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ES/ESC	embryonic stem cells
FAK	focal adhesion kinase
FDA	food and drug administration
GABA	γ-aminobutyric acid
GTP	Guanosine-5'-triphosphate
H/I	hypoxia/ischemia
HP	hypoxia preconditioning
IA	intra-arterial
IB	immunoblot
ICV	intracerebroventricular
IHC	immunohistochemistry
IL-*X*	interleukin-*X*

INA	intranasal administration
IP	intraperitoneal
iPS/iPSC	induced pluripotent stem cells
IV	intravenous
LCBF	local cerebral blood flow
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MEF	mouse embryonic fibroblast
mGluR	metabotropic glutamate receptor
MSC	mesenchymal stem/stromal cell
NeuN	neuronal nuclei
NINDS	national institute of neurological disorders and stroke
NPC	neural progenitor cell
NMDA	n-methyl-d-aspartate glutamate receptor
NSC	neural stem cell
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RMS	rostral migratory stream
RT-PCR	reverse-transcriptase PCR
SCZ	sub-callosal zone
SOD	superoxide dismutase
SGZ	sub-granular zone

SVZ	sub-ventricular zone
TNF-α	tissue necrosis factor α
tPA	tissue plasminogen activator
VEGF	vascular-endothelial growth factor
WB	western blot

Chapter 1 - Ischemic Stroke

Introduction

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Deveau TC, Yu SP, Wei L. "Cellular Therapy for Ischemic Stroke" Translational Stroke Research: From Target Selection to Clinical Trials (Springer Series in Translational Stroke Research) by Paul A. Lapchak and John H. Zhang. Springer Publishing March 23, 2012. pp. 777-817. DOI : 10.1007/978-1-4419-9530-8.

Ischemic stroke is a leading cause of death and disability in the United States. Currently, therapies for ischemic stroke are vessel and blood based (Sahota and Savitz 2011). No FDA approved treatments exist to provide neuroprotection or promote neuroprotection or central nervous system (CNS) recovery. Intense investigation into neuroprotective agents for ischemia has failed to produce a single FDA approved compound for neuroprotection following stroke. Trials involving protein therapy for recovery have faired better, with the 1995 NINDS tPA trial producing the only FDA approved drug (alteplase) for stroke therapy. Although the use of tPA clinically is a great step forward, its benefits extend to few patients in practice due to limitations of its use and its efficacy. The failure of clinical neuroprotection trials to date and the limited efficacy of thrombolytics highlight the need for not only novel treatments but also novel approaches to stroke treatment and recovery. Cellular therapy, specifically the use of stem cells for circuit replacement and/or augmentation, represents a promising new avenue for stroke therapy. The following chapter will discuss the need for novel, innovative,

treatment strategies and the current state of knowledge regarding the use of stem cells for the treatment of ischemic stroke.

Ischemic Stroke

Definition and Epidemiology

According to the World Health Organization (WHO), stroke is defined as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin" (Sacco, Kasner et al. 2013). The American Heart Association defines stroke as a disease that affects arteries feeding into and within the brain. There are two major types of stroke: ischemic and hemorrhagic. An ischemic stroke is caused by blockage of a blood vessel. Ischemic stroke accounts for the majority of stroke cases, as much as 87% in the US (Go, Mozaffarian et al. 2013). Hemorrhagic stroke, which accounts for the majority of the remaining cases, is caused by rupture of a vessel. In either case, stroke causes a lack of blood and oxygen to part of the brain that leads to the death of neurons, glia, and vascular cell components (endothelial cells, smooth muscle cells, etc) perfused by the blocked or ruptured vasculature.

Stroke is a tremendous public health concern. On average, someone in the United States has a stroke every 40 seconds. There are an estimated 795,000 new stroke cases every year. Of these cases, approximately 76.7% are new cases and the

remaining 23.2% are recurrent. Stroke is currently the 5th leading cause of death in the United States and a leading cause of disability. It is estimated that someone dies of a stroke every 4 minutes and that stroke accounts for approximately 1 in every 19 deaths. Stroke patients that survive often are faced with lifetime disabilities that require a tremendous burden of care. Disabilities faced by stroke survivors include: hemiparesis, cogitative deficits, aphasia, inability to walk without assistance, post-stroke depression and depressive symptoms. Approximately 1 in 4 stroke survivors become institutionalized in a nursing home and dependent on activities therein. The estimated cost of stroke in 2009 was $38.6 billion (Go, Mozaffarian et al. 2013).

Major risk factors for stroke include (but are not limited to): high blood pressure, diabetes, disorders of heart rhythm, physical inactivity, high blood cholesterol and other lipids, smoking, family history and genetics, and chronic kidney disease. Older adults, African-Americans, people with lower levels of education, and people living in the southeastern United States have high stroke prevalence (Go, Mozaffarian et al. 2013).

Treating stroke patients is a challenging endeavor. There is currently only one FDA approved therapy for the treatment of stroke, tissue-plasminogen activator (tPA). While tPA can be instrumental in reperfusing tissue after ischemic stroke, only a small percentage of patients are eligible for treatment. Of those that are eligible, an even smaller number receives it. There are currently no FDA-appoved therapies to improve and promote CNS repair and recovery following stroke. The

discovery of novel therapies for stroke requires a detailed knowledge of the molecular and cellular events in the brain that follow a stroke.

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Deveau TC, Yu SP, Wei L. "Cellular Therapy for Ischemic Stroke" Translational Stroke Research: From Target Selection to Clinical Trials (Springer Series in Translational Stroke Research) by Paul A. Lapchak and John H. Zhang. Springer Publishing March 23, 2012. pp. 777-817. DOI : 10.1007/978-1-4419-9530-8.

Cellular and Molecular Causes of Ischemic Injury

No discussion on therapy for stroke would be complete without some mention of causes of cellular injury following vessel occlusion and reperfusion. Investigations into stroke therapy to date have provided the scientific community with a wealth of knowledge regarding different cellular pathways and mechanisms that underlie ischemic injury. Ischemic insult initiates a complex variety of necrotic, apoptotic, and even pro-survival pathways. Depending on the time point, modulation of cellular events following ischemia provides opportunities for therapeutic intervention by cellular therapy. We present several causes of ischemic cellular injury in the following section. Others have reviewed many of these events in great detail; here we simply present a brief overview [for example, see (Lipton 1999) and (Lo, Dalkara et al. 2003) for more information]. Though presented individually, it is clear that many of these processes act in concert and play off one another. **Figure 1.1** demonstrates a time course of pathophysiological molecular and cellular events after ischemic stroke.

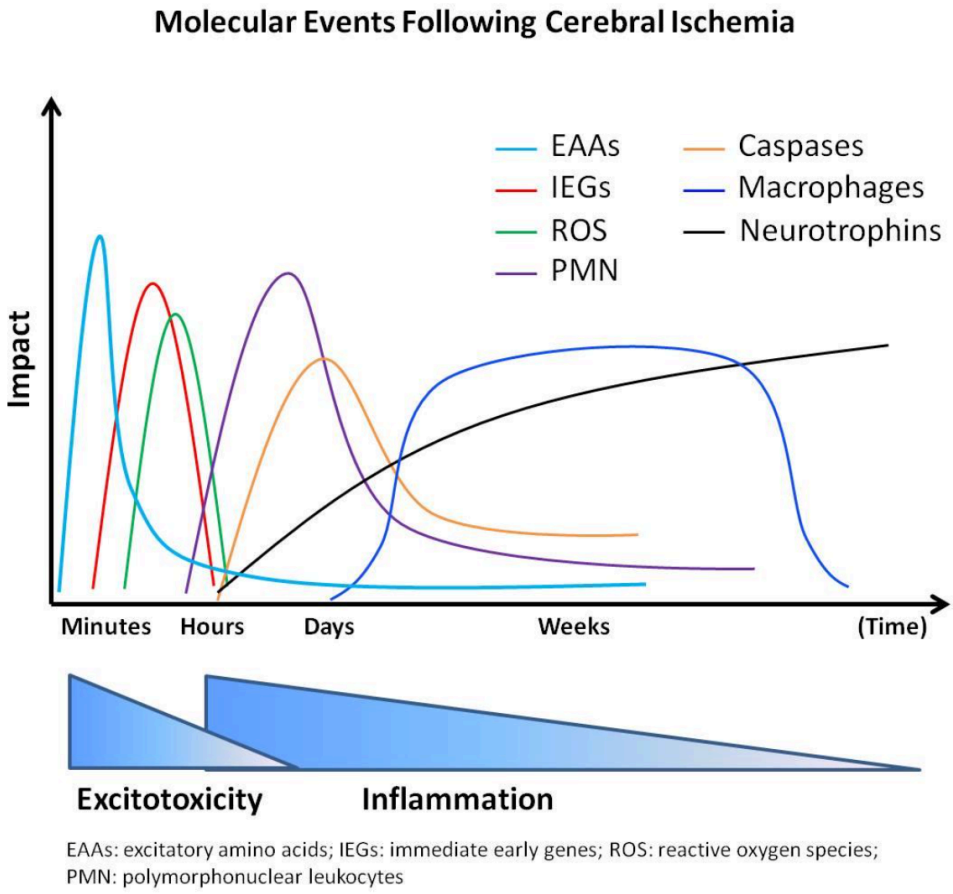

Figure 1.1 Molecular Events Following Focal Ischemia.

Within few minutes after ischemia, there is a rapid surge of excitatory amino acid release, followed by changes in gene expression and increase in ROS production. A cascade of cell death, including both apoptosis and necrosis, starts within minutes of stroke onset. Inflammation occurs causing a rise in inflammatory cytokines and leukocyte infiltration to brain parenchyma. The x-axis shows the development of the events over time, while the y-axis demonstrates the impact of each event. Figure and text courtesy of Dr. Osama Mohamad; republished with permission.

Glutamate-Mediated Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Glutamate has two types of receptors: ionotropic (AMPA, NMDA, kainate) and metabotropic (mGluR's). Under non-pathological conditions, glutamate signaling is necessary for "normal" CNS function. During ischemia, depletion of cellular energy stores leads to spontaneous "anoxic" depolarization. Large-scale anoxic depolarization in the ischemic brain in turn initiates a cascade of events that are detrimental to cells in surrounding tissue. Anoxic depolarization causes the massive release of glutamate and the overactivation of AMPA, NMDA, and kainate receptors located on surrounding neurons. Overactivation of ionotropic glutamate receptors leads to a disruption of ion gradients, specifically an excess in Na^+ and Ca^{2+} influx and K^+ efflux. Excess Na+ influx causes cellular swelling, edema, and can lead to necrosis (Lo, Dalkara et al. 2003). Excess NMDA-mediated K+ efflux during ischemia can lead to cell shrinkage and apoptosis (Yu 1999, Pal, Hartnett et al. 2003). Disruption of normal physiological ion gradients can further cause reversal of glutamate transporters, inhibiting the uptake of glutamate by glial cells, further potentiating the effects of excess glutamate release. Activation of mGluR's can be pro-death or pro-survival depending on which classes of mGluRs are activated (Bruno, Battaglia et al. 2001, Lo, Dalkara et al. 2003). The overall contribution of mGluR signaling in ischemia most likely depends on which class of mGluRs (class I, II, or III) is predominant in the injured tissue.

Excess Ca^{2+} influx is especially detrimental to cell survival, and suggested to be the primary mediator of glutamate-mediated excitotoxicity (Choi 1987, Arundine and Tymianski 2004). Under non-pathological conditions, cells are extremely sensitive to small rises in local [Ca^{2+}] and therefore Ca^{2+} is carefully buffered. Pathological intracellular [Ca^{2+}] increases lead to processes ranging from decoupling of mitochondrial electron transfer in ATP synthesis (Arundine and Tymianski 2004) to overactivation of phospholipases and a variety of other enzymes. Alterations in intracellular enzyme activity by excess Ca^{2+} influx can lead to ROS production, alterations in cytoskeletal organization, mitochondrial disfunction, and the activation of genetic signals that can lead to apoptosis (Arundine and Tymianski 2004).

Oxidative Stress

Generation of free radicals is a normal physiological process occurring in every living cell. Examples of free-radical generating processes are electron transport in the mitochondria and other enzyme mediated processes, such as synthesis of prostanoids from arachadonic acid via cyclo-oxygenases (Lo, Dalkara et al. 2003). Under non-pathological conditions, free radical scavengers such as superoxide dismutase (SOD), glutathione peroxidase, and other antioxidants are present in cells that act as an oxidative sink, preventing free radicals from causing cellular damage.

Under ischemic conditions and during reperfusion, cells in the infarct and peri-infarct tissue undergo oxidative stress. NMDA activation (Murphy, Fiskum et al. 1999, Brennan, Suh et al. 2009), intracellular ADP accumulation, excess Ca^{2+} influx, mitochondrial dysregulation, and enzymatic processes all result in the generation of excess free radicals (oxygen related superoxide, hydroxyl radicals and other nitrogen-related radicals generated from nitrous oxide) that overwhelm free radical scavengers (Lo, Dalkara et al. 2003). Excessive free radical production and resulting oxidative stress damages all molecular constituents of a cell (lipids, proteins, nucleic acids, and carbohydrates) (Lo, Dalkara et al. 2003). Oxidative stress can also damage mitochondria, causing release of mitochondrial constituents (such as cytochrome C), resulting in cellular apoptosis (Lo, Dalkara et al. 2003).

Neuroinflammation

The body of literature elucidating the importance of inflammation in the nervous system (neuroinflammation) under a variety of CNS pathologies has seen a lot of growth over the past decade. Neuroinflammation in stroke is a very complex phenomenon and not yet fully understood in relation to ischemia. Following ischemia, production of pro- and anti-inflammatory cytokines and chemokines is upregulated in endothelial cells, neurons, and glia in the brain (Lakhan, Kirchgessner et al. 2009). Additionally, ischemia causes the upregulation of extracellular matrix remodeling proteins (such as the matrix metalloproteinases or

MMPs; (Clark, Krekoski et al. 1997, Romanic, White et al. 1998), and the breakdown of the blood-brain barrier. "Leaky" regions of the blood brain barrier allow for the infiltration of peripheral immune cells such as lymphocytes, t-lymphocytes, macrophages, and neutrophils (Gelderblom, Leypoldt et al. 2009). These peripheral immune cells also contribute to cytokine production following ischemia.

Much work on inflammation in ischemia has focused on the pro-infammatory cytokines tumor necrosis-alpha (TNF-α), interleukin-six (IL-6) and interleukin one-beta (IL-1β) in addition to the anti-inflammatory cytokine interleukin-10 (IL-10). In human patients, increased IL-6 levels in serum, plasma, and CSF following cerebral ischemia have been correlated with increased neurological worsening (Vila, Castillo et al. 2000, Vila, Castillo et al. 2003, Basic Kes, Simundic et al. 2008). Increased plasma and serum IL-10 levels have been associated with less neurological worsening in humans (Vila, Castillo et al. 2000, Basic Kes, Simundic et al. 2008). Experimental studies on TNF-α and ischemia have been conflicting, with some reporting improvement with administration of TNF-α binding proteins (Nawashiro, Martin et al. 1997) and others reporting worsening with TNF-α receptor knockout (Bruce, Boling et al. 1996, Gary, Bruce-Keller et al. 1998). Experiments involving IL-1β knockout mice show that KO mice exhibit far smaller infarcts compared to wild type controls following MCAO (Boutin, LeFeuvre et al. 2001), suggesting a injurious role of IL-1β following ischemia (Lakhan, Kirchgessner et al. 2009).

Modulation of neuroinflammation may prove to be beneficial for ischemic treatment and CNS recovery. Care must be taken with conclusions from immune

research, however, as many inflammatory mediators have dual actions. TNF-α, for example, acts predominantly through the p55 (TNFR1) receptor. p55-mediated TNF-α signaling can initiate both pro-apoptotic and pro-surival intracellular cascades (Chen and Goeddel 2002, Schütze, Tchikov et al. 2008). Administration of TNF-α binding proteins can reduce ischemic injury in rats (Nawashiro, Martin et al. 1997), however ischemic injury is exacerbated in TNF receptor knockout mice (Bruce, Boling et al. 1996, Gary, Bruce-Keller et al. 1998). Microglial-derived TNF-α has also been shown to be neuroprotective (Lambertsen, Clausen et al. 2009). MMPs are another example of the dual actions of immune mediators. In the acute phase of ischemia, upregulated MMPs (such as MMP-2 and MMP-9) are detrimental to the CNS, however, in the chronic phase extracellular remodeling by MMPs are necessary for axonal growth, angiogenesis, and other repair mechanisms (Yong, Power et al. 2001). Future studies on neuroinflammation and ischemia therefore should take into account the multiplicity of inflammatory mechanisms, the temporal and spatial regulation of neuroinflammatory factors, and the consequences of the interplay of inflammation within the neurovascular unit (Lo, Dalkara et al. 2003).

Co-morbid Disorders: Post-Stroke Depression and Affective Disorders

Most research studies to date on stroke therapies have been somewhat myopic in focusing on physical CNS repair within a month or two of the stroke injury. This is understandable because physical CNS injury is the primary

pathological outcome of an ischemic stroke. Physical CNS repair and recovery is understandably the primary focus of novel therapies as a result. That being said, stroke is a very complex illness with an impact that extends beyond lack of bloodflow and cell death. Up to one-third of patients experience co-morbid depression up to 5 years after stroke, and others can experience related affective disorders like generalized anxiety disorder (Hackett and Pickles 2014). Affective disorders and co-morbities such as depression can adversely affect health-related quality of life (HRQoL) and can impede successful stroke recovery in outpatient settings (Patel, McKevitt et al. 2007).

The aim of HRQoL is to examine disease impact from the perspective of the patient. HRQoL measures can include outcomes such as physical functioning, bodily pain, general health, vitality, social functioning, emotional role, and mental health (Patel, McKevitt et al. 2007). Taken together, these outcomes and cognitive variables can be important for long-term patient recovery. Predicatively, co-morbid affective disorder presentation can negatively affect functional recovery and mortality. It can also also negatively affect cognitive function, coping responses, rehabilitation, and HRQoL after stroke as well (Morrison, Pollard et al. 2005).

As stated, most animal studies on stroke therapies focus on functional recovery. Quantitative HRQoL measures clinically are often self-reported by individuals who have suffered strokes in the form of physician-initiated questionnaires. It is difficult to assess these outcomes in rodents because frankly a communication barrier exists that prevents using the same questionnaires in exams

employed in the clinic on rodents. Although this is a problem, there are many behavioral assays that are commonly employed in rodent studies that can be substituted. For example, open-field tests can be used to assess anxiety-like symptoms in rodents after stroke; barnes and water maze tests can be used to examine long-term memory and cognitive deficits; sucrose preference tests can be employed to assess anhedonia after stroke; social approach, partition, partner preference tests, and social transmission of food tests can be used to test social interaction; ultrasonic vocalization and olfactory habituation/dishabituation tests can be used to assay communication (Silverman, Yang et al. 2010).

Cognitive dysfunction and affective disorder development following stroke are important longitudinal aspects of stroke that are often overlooked with basic research. Successful novel stroke therapies will undoubtedly improve functional recovery and have direct impacts on CNS repair and recovery after stroke. Co-morbid disorders such as post-stroke depression are very prevalent in clinical stroke populations and adversely effect long-term recovery. Consequently they should not be ignored at the basic research level. The most successful stroke therapies will address long-term co-morbid cognitive dysfunction in addition to short-term CNS repair and recovery.

Current Treatment Options: The Need for New Treatments

Every year in the United States, approximately 795,000 people have strokes. In 2007, it was estimated that the direct and indirect costs of stroke were $40.9

billion dollars and stroke was responsible for 1 out of every 18 deaths in the US (Roger, Go et al. 2011). Ischemic stroke, defined as the reduction of blood flow to an area of the brain due to vascular occlusion by the artery that supplies the affected area (Wechsler 2011), accounts for vast the majority of stroke cases (Roger, Go et al. 2011). Cerebrovascular occlusion initiates a complex cellular cascade of events, involving multiple mechanisms and pathways, which lead to irreversible tissue injury and cell death (Lo, Dalkara et al. 2003, Fisher 2011). Irreversible gray and white matter tissue injury in this context is also known as cerebral infarction. The impact of ischemic stroke is overwhelming, yet to date clinicians have few tools at their disposal for successful neurological therapy.

Despite the best efforts of clinicians and researchers worldwide, current treatments for stroke focus on prevention, clinical management of bloodflow, and rehabilitation. Timely clinical vessel recanalization and long-term rehabilitation remain the best treatment strategies for stroke patients. Recent meta-analysis of recanalization on ischemic stroke outcome suggests that timely recanalization is the most robust predictor of increase clinical prognosis following ischemia (Rha and Saver 2007, Stankowski and Gupta 2011). To date, no proven clinical treatments exist that can provide neuroprotection or promote CNS recovery.

Following cerebral ischemia, thrombolytic therapy (to be discussed in more detail in a following section) involving intravenous administration of tPA remains the best, and only, FDA approved therapy for ischemic stroke to date. Thrombolytic therapy with tPA aims to recanalize vessels through clot dissolution. tPA has many

limitations and only a small percentage of patients receive the drug in the clinic (Grunwald, Wakhloo et al. 2011). A large amount of exclusion criteria, a short temporal window for therapeutic adminstration, and risk of hemorrhage development remain major hurdles for the widespread use of thrombolytic therapy.

Advances in imaging have allowed for the increased use of endovascular therapy for mechanical clot clearance. Though not without risks, endovascular therapy offers many advantages for clot disruption over thrombolytic therapy. The use of the FDA approved devices the Merci Receiver and the Penumbra System allows for the mechanical clearance of clots. Endovascular clearance of clots often increases the rate of recanalization over thrombolytic therapy without the large risk of hemorrhage development (Gandhi, Christiano et al. 2009, Grunwald, Wakhloo et al. 2011). Although greatly beneficial for some patients, the use of endovascular therapy for stroke remains limited. The clinical infrastructure needed to successfully image the affected area and identify patients who would benefit largely impedes the widespread adoption of endovascular therapy for ischemic stroke. Additionally, incomplete removal of clots could lead to clot disruption, clot spreading, and occlusion at other sites within the brain. More research on endovascular therapy is needed (Baker, Colby et al. 2011), and, as of 2007, the AHA/ASA Stroke council only recommends the use of endovascular therapy in clinical trial settings (Adams, del Zoppo et al. 2007). A recent scientific statement on mechanical clot removal by the AHA Stroke Council states that it may be beneficial but echoes the sentiments that more research is needed (Meyers, Schumacher et al. 2009).

Rehabilitation remains the only option available to stroke patients for therapy associated with long-term recovery. Most patients survive ischemic stroke and ultimately a great health effects fall on the patients and their families due to long-term consequences of disability (Langhorne, Bernhardt et al. 2011). Despite the advances made in the clinical management of stroke, without effective medical treatments "most post-stroke care will continue to rely on rehabilitation interventions" (Kwakkel, Kollen et al. 2006, Langhorne, Bernhardt et al. 2011). There is strong evidence that suggests task-oriented training assists innate recovery following ischemia, however more research is needed on the process that underlie learning following recovery (Langhorne, Bernhardt et al. 2011). Further details of rehabilitation lie beyond the scope of the present chapter. For more information, see Miller et al 2010 and Langhorne et al 2011 (Miller, Murray et al. 2010, Langhorne, Bernhardt et al. 2011).

Translation Stroke Research: The Search for New Translational Therapies

Neuroprotection: Two Decades of Failure

Since the early 1990's, much focus on preclinical research and clinical trials has shifted to finding therapeutic agents that provide neuroprotection for ischemic tissue. Neuroprotective strategies aim to "antagonize, interrupt, or slow the sequence of injurious biochemical and molecular events that, if left unchecked,

would eventuate in irreversible ischemic damage" (Ginsberg 2009). Acute neuroprotective intervention could feasibly reduce the size of the infarction while delayed administration of neuroprotective agents would be beneficial for salvaging injured tissue within the ischemic penumbra. Any successful neuroprotective strategy would undoubtedly improve prognosis and increase the potential for and speed of long-term recovery.

A wide variety of targets have been examined for their putative neuroprotective properties. Examples of targets range from neurotransmitter receptors (AMPA, NMDA, GABA), ion channels (Na+, K+, Ca+), reactive oxygen species, cyclo-oxygenases, phospholipids, and beyond (specific details are beyond the scope of this chapter, see (Ginsberg 2008) for more detailed information). Preclinical data for many agents has been promising. Experimental evidence in animal models has demonstrated it is possible to achieve large reductions in ischemic brain injury through early intervention via neuroprotective mechanisms (Ginsberg 2009) and to improve functional outcome. Despite the preclinical evidence, no neuroprotective drug exists which has "demonstrated unequivocal efficacy in clinical trials" and "fulfilled regulatory requirements for approval" (Fisher 2011).

It is unclear whether the failure of neuroprotective agents lies in the variability in quality and reliability of preclinical data (Ginsberg 2009), shortcomings in clinical trial design, or simply failure of the preclinical agent to translate to human therapy. Regardless of the source of failure, future

neuroprotective agents will require rigorous preclinical testing, carefully designed clinical trials, and possibly even multiple mechanisms of action if neuroprotective strategies are to be successful.

Examples of issues surrounding the quality of preclinical data for many putative neuroprotective agents to date include incomplete pharmacokinetics data, lack of an adequate dose-response curve, poorly defined therapeutic window, and failure to test compounds in a variety of models with a variety of confounding factors (Fisher 2011). Doubt has even been cast on the validity of animal models for ischemic stroke. Evidence, however, suggests that animals in animal models undergo similar physiological changes to humans throughout ischemia and reperfusion, suggesting that animal models should have predictive validity for evaluating neuroprotective agents (Richard Green, Odergren et al.). In an effort to improve the quality of preclinical data, the STAIR guidelines have been proposed. First outlined in 1999 [STAIR Roundtable (1999)] and recently updated (Fisher, Feuerstein et al. 2009), the STAIR guidelines provide researchers with a checklist of data that should to be generated before a putative neuroprotective agent should go to clinical trials. The effect of the STAIR trial guidelines on the translational success of preclinical neuroprotective agents is yet to be determined (Ginsberg 2009, Fisher 2011).

Confounding factors of the trials themselves bears some of the burden of the state of neuroprotection trials to date. Inherent problems with study design have impeded the ability of clinical trials to reach appropriate conclusions in some

instances. The proper timing of drug delivery for efficacy, sample sizes, heterogenous patient populations, and lack of penumbral imaging are examples of the issues that have surrounded clinical trials for neuroprotection to date (Fisher 2011). Future clinical trials will require careful design and rigorous design adherence in order to adequately determine any potential benefit of putative neuroprotective agents.

Another potential underlying factor in the failure of neuroprotection trials to date is that most neuroprotective agents investigated act on one pathway or have one mechanism of action. Although neuroprotection trials have failed in bringing neuroprotective agents into widespread use, their impact overall is not negative. Numerous trials to date have elucidated the roles of many different pathways in the context of ischemia. For example, we now know ischemic cell death involves glutamate-mediated excitoxicity through anoxic depolarization, disruption of ion and neurotransmitter gradients, aberrant intracellular Ca^{2+} signaling, oxidative stress and ROS, intrinsic and extrinsic neuroinflammation, protease induction, DNA damage through caspase activation, death of different cell types, and many other factors (Lo, Dalkara et al. 2003). Through animal models and the failure of clinical trials, we have learned stroke is a very heterogenous injury that affects very heterogenous populations. Successful therapies for stroke will therefore need to involve multiple mechanisms to account for disease heterogeneity, and a need exists to investigate therapies with multiple mechanisms of action.

Thrombolytic Research: Thrombolytics and tPA

The use of thrombolytic agents to treat coronary occlusion in the cardiovascular field has been widespread for decades. Because the vast majority of strokes are ischemic in nature (~85%), thrombolytic agents used in the cardiovascular field inevitably found a place in the treatment of ischemic stroke as well (Barreto 2011). Initial studies focused on the use of urokinase and streptokinase, but studies utilizing intravenous tPA finally provided a breakthrough for acute ischemic stroke threrapy. Newer studies on synthetic thrombolytics and xenogenic thrombolytics are currently underway. These newer thrombolytic agents aim to improve the speed of recanalization and to extend the time window for treatment. Unfortunately thrombolytic therapy also carries a large risk of hemorrhage development, a hurdle that must always be taken into account when researching new thrombolytic therapies. Although thrombolytic therapy for stroke has provided huge benefits for some patients, its application is limited, again highlighting a need for novel approaches.

Early clinical trials for thrombolytic therapy and ischemic stroke focused on the use of streptokinase (Blakeley and Llinas 2007). Trials involving streptokinase demonstrated its efficacy in acute myocardial infarction in the 1970's and 1980's (Barreto 2011). The use of streptokinase moved towards the treatment of cerebral ischemia in the 1980's and 1990's. Case reports demonstrated the efficacy of streptokinase in recanalization however use was cautioned due to risks of hemorrhage. The MAST-I [MAST I Group (1995)], MAST-E [MAST E Group (1996)],

and ASK (Donnan, Davis et al. 1996) trials in the 90's examined the use of streptokinase but were terminated early due to unacceptable increases in patient mortality (Hommel, Boissel et al. 1995, Blakeley and Llinas 2007).

To date, the NINDS tPA trials in the mid-90's have been the only Phase III clinical trials to produce an FDA approved therapy for stroke. These trials found that patients receiving intravenous administration of tPA within 3 hours of ischemic onset were 30% more likely to exhibit minimal or no disability compared to placebo 90 days after drug administration [NINDS Study Group (1995, Gandhi, Christiano et al. 2009). Recent research on tPA has focused on extending the therapeutic window of tPA administration. The European ECASS-III trial formally demonstrated that tPA can benefit some patients if the time window is extended from 3 hours to 4.5 hours (Hacke, Kaste et al. 2008). The results of the ECASS-III trial and the Australian EPITHET trial (Davis, Donnan et al. 2008) resulted in the AHA/ASA Stroke Council changing their stance on tPA use and extending the recommended time window to 4.5 hours in some patients (Del Zoppo, Saver et al. 2009).

Further research has examined the use of new thrombolytics. Newer agents aim to increase thrombolytic efficacy by providing longer half-lives and/or greater specificity for clot components. Tenecteplase and desmoteplase are two examples of so-called "third generation" thrombolytics currently being tested in early clinical trials (Wechsler 2011). Tenecteplase is a modified form of tPA designed to increase half life and fibrin specificity and desmoteplase is a venom from vampire bat saliva (Barreto 2011). Data from preclinical work and early trials is currently mixed with

further study on these compounds needed before they can be recommended for therapy for ischemic stroke (Wu and Grotta 2010, Barreto 2011, Wechsler 2011).

While the use of thrombolytics has been groundbreaking, thrombolytic therapy is not without limitations. Largely as a result of the short window of time in which treatment can be administered, it is estimated that only as many as 3-4% of eligible patients throughout the US and Europe receive tPA (Grunwald, Wakhloo et al. 2011). Additionally, many patients simply are not eligible for thrombolytic therapy due a large amount of exclusion criteria. Current research examining intra-arterial (IA) administration, the effects of combinatorial thrombolytic administration and pharmacotherapy, and thrombolytic administration plus endovascular treatments are ongoing. Whether or not IA thrombolysis or combinatorial thrombolytic treatment will increase recanalization rates, extend time windows, and decrease risks of hemorrhage are yet to be seen.

Summary : The Need for Novel Approaches and Cellular Therapy

Current treatment options for stroke highlight the need for novel treatments and innovative strategic interventions to improve patient prognosis. The failures of research to date (other than tPA) to generate FDA approved treatments that promote CNS neuroprotection or recovery demonstrate the need for new approaches to treating stroke. A potential new approach to stroke treatment lies in cellular therapy. Interest in the use of stem cells for the treatment of CNS disorders

and CNS injury has been growing since the 1980's, when the first cells were transplanted into Parkinsons patients. Since initial Parkinsons trials, cell transplants have been used in other CNS disorders, such as Huntingtons, and even ischemic stroke itself.

Stem cell therapy provides a unique approach to brain repair and functional recovery in the post-ischemic brain (Sahota and Savitz 2011). Stem cells hold great promise for stroke treatment because cellular therapy has multiple mechanisms of action, can respond to changes in the surrounding cellular microenvironment, and substantially increases the time window within which treatment can be administered. Cellular therapy targets aspects of the repairing nervous system by modulating innate, endogenous, neurogenesis or providing exogenous cells for cellular replacement and/or augmentation (Sahota and Savitz 2011).

The remainder of the chapter will be devoted to cellular therapy for stroke. From the source of cells, putative mechanisms of action, and the timing and delivery method of cells, we will discuss the state of knowledge regarding cellular therapy for stroke. Reviews such as this aim to provide investigators with the tools to direct their research and advance the field of cellular therapy, not only for stroke, but also for other CNS disorders as well.

Chapter 2 - Cellular Therapy: Stem Cells, iPS Cells, and Cell Transplantation

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Deveau TC, Yu SP, Wei L. "Cellular Therapy for Ischemic Stroke" Translational Stroke Research: From Target Selection to Clinical Trials (Springer Series in Translational Stroke Research) by Paul A. Lapchak and John H. Zhang. Springer Publishing March 23, 2012. pp. 777-817. DOI : 10.1007/978-1-4419-9530-8.

Post-Ischemic Cellular Therapy: The Next Frontier

The past two decades has seen an explosion of research on the use of stem cells as a therapy for ischemic stroke. Embryonic stem cells are often the first cell type that comes to mind however; from the literature it is clear that other sources of non-embryonic stem cells exist. Non-embryonic cells can be isolated from bone marrow, umbilical cords, and even the adult CNS. Embryonic and non-embryonic cells isolated from any of these sources all carry therapeutic potential for CNS disorders, especially ischemic stroke. Additionally, it is now clear that neurogenesis in the SGZ and SVZ is regulated by ischemia, and increasing the proliferation and survival of adult neural progenitor cells in these regions may provide therapeutic benefit. Cells from different sources do not necessarily have the same properties, and an understanding of the properties of different stem cells from different sources is necessary when considering which stem cell type to use for stroke therapy.

Cellular Therapy: Sources of Cells

Cellular therapy can be divided in to exogenous and endogenous therapy. Exogenous cellular therapy involves the transplantation of xenogenic, allogenic, or autologous cells through a variety of means. Endogenous therapy involves the stimulation or amplification of innate neurogenesis in the ischemic brain through extrinsic and/or intrinsic factors. We will now consider different sources of cells for cellular therapy, different mechanisms of action, and other considerations regarding the use of cellular therapy for ischemia.

Endogenous NPCs, Neurogenesis, and NPC Migration

Dating back to Cajal in the early 20th century, prevailing dogma thought that no new cell growth occurs in the adult CNS. However, early 20th century views on cell division and growth in the adult brain were limited by the technology of the time. Since Cajal's time, tools like [H^3]-thymidine and Brdu have come along that have made the study of adult neurogenesis possible (Rakic 2002). In 1962, Joseph Altman published the first study challenging canonical thinking regarding neurogenesis in the adult mammlian brain (ALTMAN 1962, Wiltrout, Lang et al. 2007). Using radiolabeled thymidine to mark dividing cells, he noticed neuroblasts and even neurons incorporated the radiolabeled thymidine following electrolytic lesions to the lateral geniculate body. Early studies on neurogenesis were met with skepticism. In 1977, Kaplan and Hinds provided the first concrete evidence of neurogenesis with electron microscopy in 90-day-old rats. They showed cells in the dentate gyrus and olfactory bulb that incorporated radiolabeled thymidine were

indeed neurons (Kaplan and Hinds 1977, Wiltrout, Lang et al. 2007). Acceptance of neurogenesis in adult vertebrates was further solidified by work done in the songbird field in the early 1980s by Nottebohm and Goldman (Nottebohm 1981, Goldman and Nottebohm 1983, Wiltrout, Lang et al. 2007). Since then, adult neurogenesis has been demonstrated in many species, including non-human primates (Gould, Reeves et al. 1999) and humans (Eriksson, Perfilieva et al. 1998).

We now know unequivocally that new proliferating cells are born every day in several restricted niches in the adult mammalian CNS: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, the subventricular zone (SVZ), and the recently described subcallasal zone (SCZ). Neurogenesis in these areas is regulated by a variety of CNS pathologies in what appears to be an attempt at self-repair. While the innate neurogenic response to ischemia seems incapable of being functionally efficacious, bolstering the mechanisms surrounding this response may be a potential therapy for ischemic stroke.

Neurogenesis in the Sub-Granular Zone (SGZ)

The subgranular zone of the hippocampus was the first region in the adult mammalian brain where dividing neurons were concretely identified (Altman and Das 1965, Kaplan and Hinds 1977). Neurogenic cells in the SGZ have a limited capacity to self renew (Wiltrout, Lang et al. 2007) and it is debated whether or not cells in the SGZ truly meet the full criteria of “stem cells” (Burns, Verfaillie et al.

2009). The exact identity of progenitor parent population in the SGZ is unclear, however progenitor cells express GFAP and are postulated to be radial glial cells or the remnants thereof (Gould, Vail et al. 2001, Wiltrout, Lang et al. 2007). Nevertheless, it is estimated that approximately 9,000 new cells are generated in the hippocampus of the adult rat everyday (Cameron and McKay 2001) and lower numbers in the mouse that appear to vary by strain (Hayes and Nowakowski 2002, Christie and Cameron 2006). The numbers in the human hippocampus are not known, but it is known that hippocampal neurogenesis does occur in humans (Eriksson, Perfilieva et al. 1998). SGZ-derived proliferating progenitors ultimately migrate to the granule cell layer (GCL) where the majority become excitatory granule neurons. A smaller population, observed at about 14% of the total proliferating population in adult transgenic rats, becomes GABAergic basket cells (Liu, Wang et al. 2003). Further studies are needed to determine the mechanisms by which newly proliferating cells become granule neurons or GABAergic neurons, but evidence suggests that progenitors form a somewhat heterogenous neuronal population in the GCL (Lledo, Alonso et al. 2006).

Much work has been done to characterize newborn cells in the GCL. In rodents, new cells have been shown to receive synaptic input, express neuronal markers, and extend processes into the CA3 region of the hippocampus (Gould, Vail et al. 2001). Proliferating SGZ-derived neural progenitors mature, migrate, and differentiate in a stepwise fashion. Immature proliferating granule cells first express the markers DCX and PSA-NCAM and show expression of GABA, AMPA, and NMDA receptors (Lledo, Alonso et al. 2006). As they mature, DCX and PSA-NCAM

marker expression fades, giving way to NeuN and Calretinin expression with a growth in excitatory GABAergic synaptic input (Lledo, Alonso et al. 2006). NeuN and Calretinin positive immature granule cells exhibit small, minimally repetitive, action potentials (Lledo, Alonso et al. 2006). Mature adult SGZ-derived new granule neurons are mature at around 28 days after birth in the rodent. Mature new granule cells express the markers NeuN and Calbindin; express GABA, AMPA, and NMDA receptors; receive synaptic input from GABA receptors, AMPA receptors, and NMDA receptors; last mature newborn granule neurons display large, repetitive, action potentials (Lledo, Alonso et al. 2006). Although in rodent models granule cells are mature at around 28 days, a recent study by Kohler and colleagues (2011) suggests that in primates maturation time is longer, exceeding 6 months (Kohler, Williams et al. 2011).

A large percentage of new-born granule cells die in the GCL within 28 days of age (Gould, Vail et al. 2001, Dayer, Ford et al. 2003). Neurogenesis in the SGZ persists throughout life, however there is an age-dependent decrease throughout the life of animals (Kuhn, Dickinson-Anson et al. 1996). The exact function of new neurons in the GCL from SGZ-derived progenitors is unclear. Strong evidence suggests that SGZ neurogenesis plays an important role in learning and memory, however a definitive causal relationship has remained elusive (Burns, Verfaillie et al. 2009). Animal models in which proliferating cells in the adult SGZ can be selectively ablated will be needed in order to establish definitively the role of SGZ neurogenesis.

Neurogenesis in the Subventricular Zone (SVZ)

Cortical neurons in the embryonic rodent arise from neural stem cells in the ventricular zone (VZ) (Zhang, Zhang et al. 2008). Following embryonic cortical neurogenesis, an ependymal layer replaces the ventricular zone, but the subventricular zone (SVZ) persists throughout life (Morshead, Craig et al. 1998, Zhang, Zhang et al. 2008). A subpopulation of radial glia from the VZ transform into adult astrocytes in the SVZ that form the basis of neural stem cells in the adult SVZ (Tramontin, García-Verdugo et al. 2003, Bonfanti and Peretto 2007, Zhang, Zhang et al. 2008). Each day, more than 30,000 neuroblasts are generated in the adult SVZ (Alvarez-Buylla, García-Verdugo et al. 2001, Lledo, Alonso et al. 2006, Zhang, Zhang et al. 2008). SVZ-derived neuroblasts form chains that are ensheathed by astrocytes and migrate relative great distances through the rostral migratory stream (RMS) towards the olfactory bulb under non-pathological conditions (ALTMAN 1969, Alvarez-Buylla, García-Verdugo et al. 2001, Lledo, Alonso et al. 2006, Zhang, Zhang et al. 2008, Burns, Verfaillie et al. 2009). In the olfactory bulb, these cells differentiate into granule and periglomular interneurons (Lledo, Alonso et al. 2006).

Doestch and colleagues performed ultrastructural studies on the cellular composition of the SVZ in 1997 (Doetsch, García-Verdugo et al. 1997). Type A, B, C, D, and E cells are all present in the SVZ as confirmed by electron microscopy and serial reconstruction of ultrathin sections. Type A cells represent migrating

neuroblasts, type B cells represent astrocytic parent cells, type C cells putative neural precursors, type D tanycytes, and type E large ependymal cells (for a detailed description of morphological characteristics of different cell types based on electron micrographs, please see (Doetsch, García-Verdugo et al. 1997)). Type A, B, and C cells all incorporated [H^3]-thymidine in the SVZ of adult mice, suggesting that all three cell types are dividing cells.

SVZ-derived granule and periglomular neurons differentiate and mature in a stepwise fashion similar to SGZ-derived granule neurons. SVZ-derived granule cells are mature around 14 days after birth in the rodent (Petreanu and Alvarez-Buylla 2002, Lledo, Alonso et al. 2006). Immature granule cells express the markers DCX and PSA-NCAM which subsequently gives way to TUJ1 + TUC-4 and finally NeuN expression throughout maturation. Granule cells express GABA, AMPA, and NMDA receptors and receive synaptic input from GABA, AMPA, and NMDA receptors. Sodium currents are observed only after synaptic contacts are established. SVZ-derived periglomular cells take longer to mature, around 28 days in the adult rodent. Immature periglomular cells first express the markers DCX and PSA-NCAM which gives way to TUJ1 + TUC-4 expression, followed by NeuN + GAD/Calbindin/Calretinin expression, and finally TH expression sequentially through maturation. They express GABA, AMPA, and NMDA receptors. Synaptic input is first provided by GABA neurotransmission about mid-way through maturation, followed by AMPA and NMDA mediated inputs. Periglomular cells exhibit sodium currents before synaptic inputs are made. (Lledo, Alonso et al. 2006). It is suggested that as many as 50% of SVZ-derived granule cells that reach the

olfactory bulb die and that sensory input is critical for those that survive after 15 days (Petreanu and Alvarez-Buylla 2002).

SVZ neurogenesis has been best studied and characterized in rodents (Burns, Verfaillie et al. 2009). Evidence exists for the generation of new neurons and the RMS in non-human primates (Pencea, Bingaman et al. 2001, Wang, Liu et al. 2011) and in the adult human brain (Curtis, Kam et al. 2007, Wang, Liu et al. 2011). Characterization of SVZ-NPCs and the RMS in humans has remained controversial (Sanai, Berger et al. 2007, Burns, Verfaillie et al. 2009), although a recent study by Wang et al (2011) seems to support initial observations by Curtis and colleagues that dividing cells are present in the adult human SVZ and migrate towards the olfactory bulb in the RMS (Wang, Liu et al. 2011). The functional role of SVZ neurogenesis in rodents is yet to be definitely defined but "correlative evidence" suggests "a role in olfactory memory and discrimination" (Burns, Verfaillie et al. 2009).

Following ischemia in animal models, SVZ-derived NPCs migrate laterally away from the RMS towards infarcted tissue by way of chemotaxic gradients such as the SDF-1α and CXCR4 axis (to be discussed in more detail in the following section). Migration of SVZ-derived NPCs towards injured tissue following ischemia suggests an attempt at self-repair by the adult brain, however a large majority of migrating cells die (Arvidsson, Collin et al. 2002). It is yet to be seen if NPCs from the SVZ can make a meaningful contribution to neuronal recovery following ischemia. That said, a much greater number of neuroblasts are generated than survive (Arvidsson, Collin

et al. 2002), suggesting the potential that migration of SVZ-derived NPCs have for stroke therapy.

Neurogenesis in the Subcallosal Zone (SCZ)

Most discussions on neurogenesis in the adult mammalian brain have focused mainly on the SGZ and the SVZ. Evidence for neurogenesis has been described in other areas of the brain such as the neocortex (Rakic 2002) and the substantia nigra (Zhao, Momma et al. 2003), but whether or not neurogenesis truly occurs in these regions remains controversial. One recently characterized region where neurogenesis appears to occur is the subcallosal zone (SCZ). Anatomically, the SCZ lies between the hippocampus and the corpus callosum (CC) in the adult rodent. It extends medial and caudal from the SVZ and is comprised of a "lamina of proliferating cells" that is "sandwiched" between axon bundles of the hippocampus and CC (Seri, Herrera et al. 2006).

Cells in the SCZ have similar properties to those of the SVZ, and are comprised of type E ependymal cells, type A migrating cells, type B astrocytes, and type C dividing cells (Doetsch, García-Verdugo et al. 1997, Seri, Herrera et al. 2006). In culture, they can form neurospheres, and SCZ-derived NSCs can differentiate into neurons, astrocytes, and oligodendrocytes. *In vitro* experiments suggest that cells from the SCZ form "multipotent, self-renewing" stem cells. SCZ-derived cells differ from SVZ-derived cells in that they form less primary neurospheres in culture and

form isolated PSA-NCAM positive clusters, unlike the PSA-NCAM positive elongated chains that cells from the SVZ form (Seri, Herrera et al. 2006).

The exact function of SCZ-derived NPCs and their potential to contribute to self-repair is unknown. *In vivo* microtransplantation experiments using GFP-labeled SCZ cells from adult β-actin-GFP mice transplanted into the SCZ of adult CD1 mice suggest that SCZ-derived NPCs become Rip-1 positive oligodendrocytes and GFAP positive astrocytes that migrate into the CC (Seri, Herrera et al. 2006). A recent study by Kim and colleagues (2011) suggests that neurons are formed by SCZ-NPCs, but these newborn neurons undergo massive Bax-mediated programmed cell death (Kim, Chun et al. 2011). Interestingly, a 2002 study by Nakatomi and colleagues showed that proliferation was increased the pPV (area within the recently described SCZ) following ischemia in rodents and that pPV-derived cells contributed to neuronal replacement in the CA1 region of the hippocampus, a process that can be amplified with ICV growth factor infusion (Nakatomi, Kuriu et al. 2002). While it seems that the SCZ has the potential to contribute to self-repair after ischemia, more studies are needed to determine the role of SCZ-derived NPCs and ischemia and the capacity of SCZ-NPCs to contribute to neuronal self-repair.

Exogenous Cells

The nervous system is a complex organ made up of neurons and glial cells, which surround and support neurons (Ming and Song 2005, Ming and Song 2011). Neurons send signals that provide the driving force (in one way or another) for

numerous functions including, but not limited to, thought processes and movement. Glial cells, including astrocytes, microglia, and oligodendrocytes are necessary for the normal function of the nervous system. For example, oligodendrocytes act to enhance the conduction velocity of neural signals. Neural signals, such as descending motor commands into the spinal cord, sometimes must traverse great distances. Support from oligodendrocytes is necessary for efficient neural signal transduction across time and space. The loss of any of these cell types may have grave implications for brain function. Most of the advances in stem cell research to date have been directed at treating degenerative diseases through cellular replacement. Following ischemic stroke, multiple cell types are lost in the infarcted region. Cellular therapy for ischemic stroke holds promise in the potential for cell replacement across different cell types, although research shows stem cells can also exert therapeutic benefit by providing support for injured neurons and glia in the peri-infarct region. Whether aiming for cellular replacement or cellular augmentation, cellular therapy holds great promise for the treatment of ischemic stroke. Currently, stem cell research plays an important role in pushing the boundries for therapy of neurological disorders (Lindvall and Kokaia 2006, Orlacchio, Bernardi et al. 2010, Shimada and Spees 2011).

Neural stem cells (NSCs)

Beginning with work done published by Joseph Altman in the 1960's (ALTMAN 1962, Altman and Das 1965, ALTMAN 1969), it is well established that multipotent neural stem cells (NSCs) exist in several regions of the brain that have the capacity to self-renew. The three niches of neurogenesis in the adult mammalian brain are the subventricular zone (SVZ) lining the leteral ventricle, the subgranular zone (SGZ) in the dentate gyrus of hippocampus (Ming and Song 2005, Ming and Song 2011), and the subcallosal zone (SCZ), which lies between the corpus collasum and hippocampus (Seri, Herrera et al. 2006). New neurons and glia are generated from these areas and are thought to contribute to neuronal plasticity, possibly even neuronal repair following CNS injury (Lledo, Alonso et al. 2006). NSCs from the adult mammalian brain have the potential to differentiate into astrocytes, neurons, and oligodendrocytes (Taupin 2006). The three neurogenic niches in the adult mammalian CNS will be described in more detail in the following section.

The behavior of NSCs is controlled by many intrinsic and extrinsic factors (Lie, Song et al. 2004, Hsu, Lee et al. 2007, Massirer, Carromeu et al. 2011). An example of instrinsic regulation of NSC differentiation and proliferation is the binding of transcription factors, such as the neuron-restrictive silencing factor/RE-1 silencing transcription (REST) factor system and retinoic acid (Kazanis, Lathia et al. 2008, Massirer, Carromeu et al. 2011). Transcription factors exert changes in the transcriptome profiles of NSCs, changes that are tightly associated with NSC "state" (Massirer, Carromeu et al. 2011). DNA modifications themselves are also known to affect NSC behavior. For example, chromatin remodeling and associated changes in methylation state of CpGs affect gene expression patterns in NSCs *in vitro* (Kazanis,

Lathia et al. 2008, Massirer, Carromeu et al. 2011). In addition to intrinsic factors, extrinsic factors can also regulate behavior of NSCs (Hsu, Lee et al. 2007, Qu and Shi 2009, Massirer, Carromeu et al. 2011). For example, when NSCs are exposed to hypoxia, hypoxia itself can modulate NSC differentiation and proliferation (De Filippis and Delia 2011). Growth factors, such as EGF and FGF-2, are another example of extrinsic factors that also enhance the proliferation and differentiation of NSCs (Kazanis, Lathia et al. 2008, Massirer, Carromeu et al. 2011).

Neurogenesis in the adult mammalian brain is enhanced following CNS injury, and new neuronal cells are generated and migrate to the sites of injuries (Imitola, Raddassi et al. 2004, Pluchino, Zanotti et al. 2005, Taupin 2006, Zhang, Zhang et al. 2008, Sahota and Savitz 2011). The contribution of endogenous adult neurogenesis to non-pathological and pathological CNS function is currently unclear. Correlations have been seen with hippocampal neurogenesis and learning (Burns, Verfaillie et al. 2009). Potential mechanisms behind putative increases in cognition as a result of adult hippocampal neurogenesis include computational theories (Becker 2005), reduced interference between memories (Wiskott, Rasch et al. 2006), and as a mechanism to encode temporal aspects of memory (Aimone, Wiles et al. 2006). Despite correlative evidence, a clear causal relationship between adult neurogenesis and learning and memory remains elusive (Lie, Song et al. 2004, Ming and Song 2005, Jordan, Ming et al. 2006, Burns, Verfaillie et al. 2009, Ming and Song 2011).

NSCs can be isolated from embryonic, fetal, and adult mammalian brains and cultured *in vitro* as neurospheres (Massirer, Carromeu et al. 2011). *In vitro*, neurospheres from NSCs provide a tool with which to study neurogenesis. NSC neurosphere cultures can also be expanded *in vitro* and used for transplantation studies. NSCs *in vitro* have the capacity to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes (Bithell and Williams 2005). NSC transplantation has been studied in several animal models of CNS pathologies, such as Parkinson's Disease (PD), multiple sclerosis (MS), cerebral ischemia, and intracerebral hemorrhage (ICH) (Bithell and Williams 2005, Pluchino, Zanotti et al. 2005). In animal models, adult NSC transplantation can aid in neural repair (Bithell and Williams 2005, Pluchino, Zanotti et al. 2005). NSCs show a lot of promise for ischemic stroke therapy and can easily be differentiated into neurons and glia experimentally. However, NSC sources lie deep in the brain, which clinically favors the use of other cell types that are more accessible over NSCs.

Mesenchymal stem cells (MSCs)

Friedenstein et al first described mesenchymal stem cells (MSCs) derived from bone marrow in the 1960's (Friedenstein, Piatetzky et al. 1966, Friedenstein, Petrakova et al. 1968, Bernardo, Locatelli et al. 2009). MSCs, also known as "multipotent mesenchymal stromal cells" (Horwitz, Le Blanc et al. 2005), have since been found in other tissues and other sources including periosteum, muscle

connective tissue, umbilical cord blood, placenta and amniotic fluid, adipose tissue, and fetal tissue (Bernardo, Locatelli et al. 2009, Malgieri, Kantzari et al. 2010). As defined in 2005, the minimal criteria for a cell to considered a multipotent human mesenchymal stromal cells is: 1) cell must be adherent to plastic under standard culture conditions; 2) exhibit positive expression of the cell surface markers CD105, CD73, and CD90 and be negative for hematopoietic markers CD34, CD45, CD11a, CD19, HLA-DR; 3) differentiate into adipocytes, osteocytes, and chondrocytes *in vitro* under specific conditions (Horwitz, Le Blanc et al. 2005, Salem and Thiemermann 2010). MSCs represent a small fraction of nucleated cells in the parent tissue. It is estimated that in bone marrow, MSCs represent around 1 in 10,000 nucleated cells in a newborn, declining to around 1 in 1,000,000 by age 80 (Caplan 1994, Bernardo, Locatelli et al. 2009). Despite the relatively low abundance of MSCs in mammlian tissue, MSCs can be isolated and expanded *in vitro*.

In vitro, MSCs in culture rapidly self-renew and can be passaged for high numbers, although they are not immortal (Kemp, Hows et al. 2005). MSC cultures are considered a heterogenic mix of committed and uncommitted progenitors. Cells in culture generally have the potential to form multiple mesenchymal cell types, with some progenitors capable of multilineage differentiation that can form endodermal and ectodermal cells (Kemp, Hows et al. 2005). Evidence shows that MSCs can be differentiated into neurons, however this is far from conclusive due to cell fusion and spontaneous expression of neuronal markers by undifferentiated cells (Xu, Miki et al. 2010, Thomas, Stone et al. 2011).

Despite the controversy surrounding differentation of MSCs into functional neurons (Thomas, Stone et al. 2011), MSCs may not need to differentiate into functional neurons to exhibit therapeutic benefits. Intravenous MSC-NPC transplantation has been tested extensively in animal models of cerebral ischemia and shown to home to the ischemic region through chemokine-chemokine receptor interactions (Rosenkranz, Kumbruch et al. 2009), providing benefit for repair and functional recovery. Although the precise mechanisms behind IV-MSC transplantation are not yet fully understood, many studies show benefits of MSC transplantation through potential paracrine mechanisms such as: angiogenesis (Wu, Chen et al. 2007), trophic factor expression (Wakabayashi, Nagai et al. 2010), and immune modulation (Li, Zhu et al. 2010). Additionally, clinical trials have also been performed with IV transplanted MSCs following ischemia. Trials to date demonstrate not only the feasibility of IV transplantation of autologous MSCs, but also that the approach is clinically safe and may provide some benefit for functional recovery (Lee, Hong et al. 2010). Last, although most transplantation studies to date have focused on intravenous MSC transplantation, other transplantation routes with MSCs have shown benefit in animal models. For example, intracerebral human MSC transplantation has also provided benefit in gerbils and non-human primates following ischemia (Li, Zhu et al. 2010, Xu, Miki et al. 2010)

Most work on MSCs to date has been done in bone marrow derived MSCs. More concrete studies are needed on their ability to generate functional cells of the nervous system. Additionally, due to the fact that abundance decreases with age along with their proliferation/differentiation capacity, and the fact that bone

marrow cells must be obtained through a painful and invasive procedure, more studies on the use of alternate sources of MSCs for ischemic stroke therapy (Malgieri, Kantzari et al. 2010).

Embryonic stem cells (ESCs)

Due to the ethical, moral, and political implications surrounding their harvest and use, embryonic stem cells are most often what comes to mind when stem cells are discussed. Embryonic stem cells (ES cells) are derived from the inner cell mass (ICM) of the blastocyst (an early-stage embryo) from a variety of sources, including mouse, non-human primates, and humans (Thomson, Kalishman et al. 1995, Thomson, Itskovitz-Eldor et al. 1998, Odorico, Kaufman et al. 2001). Thomson and colleagues first established human ES (hES) cell lines in 1998. ICMs of several human embryos produced for clinical purposes through *in vitro* fertilization (IVF) were isolated and expanded/maintained in culture indefinitely, providing researchers with H1, H7, H9, H13, and H14 hES cell lines still in use today (Thomson, Itskovitz-Eldor et al. 1998). hES cells are distinct from other embryonal pluripotent cells, such as teratocarcinoma-derived pluripotent embyronal carcinoma (EC) cells (Thomson, Itskovitz-Eldor et al. 1998). hES cells are pluripotent and are able to differentiate into all cellular derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm (Odorico, Kaufman et al. 2001). hES cells are powerful cells with much potential for CNS therapy. Serious

moral and ethical issues are raised with the isolation of hES cells as collection of the ICM destroys the blastocyst and the fertilized embyro (Chu 2003, Devolder 2005, Jain 2005). Additionally, hES cells are surrounded by political controversy in the US, as exemplified by the recent ruling of Chief Justice Lambert of the District of Columbia Federal District Court in August of 2010 who put an injunction on the use of federal funding for hES research (Harris 2010).

Grafted ES cells have been seen to differentiate into neurons and functionally integrate into the recipient brain in several models of CNS pathologies, including Parkinsons and stroke (Bjorklund, Sanchez-Pernaute et al. 2002, Buhnemann, Scholz et al. 2006, Guillaume, Johnson et al. 2006, Zhang, Li et al. 2009). *In vivo* studies have shown benefit of ES transplantation specifically for stroke as well. Transplantation of neural precursors derived from ES cells improved functional recovery in models of focal and global ischemia in rats (Wei, Cui et al. 2005, Daadi, Maag et al. 2008, Takahashi, Yasuhara et al. 2008, Theus, Wei et al. 2008, Hicks, Lappalainen et al. 2009, Kang, Kim et al. 2010). Although ES cells have shown benefit in animal models of ischemia, current transplantation approaches may be sub-optimal due to the inherently low survival of cells as a result of the toxic post-ischemic microenvironment in the peri-infarct cortex. Pre-transplantation cellular modifications have been demonstrated to improve therapeutic potential of transplanted ES cells, ostensibly through increased graft survival. For example, over-expression of the antiapoptotic gene bcl-2 and exposure to sub-lethal hypoxic insults before transplantation has been shown to improve the benefits of ES transplantation for ischemia (Wei, Cui et al. 2005, Theus, Wei et al. 2008). Futher

studies should examine the optimization of ES cell transplantation for ischemic stroke.

Research to date on the use of ES cells for CNS recovery following ischemia is promising. The use of ES cells of any origin has advantages over other cell types relating to their capacity for multilineage differentiation and self-renewal ability. These properties do not come without a price and the use of ES cells carries a high risk of teratoma formation (for clarification of teratoma nomenclature, please see (Damjanov and Andrews 2007), "hindering the fufillment of the clinical potential" (Ben-David and Benvenisty 2011). Several studies have shown that ES cell grafts develop teratomas when transplanted into severely combined immunodeficient (SCID) mice, regardless of the species cells were derived from (Thomson, Itskovitz-Eldor et al. 1998, Asano, Sasaki et al. 2006, Shih, Forman et al. 2007, Seminatore, Polentes et al. 2010, Ben-David and Benvenisty 2011). The post-ischemic environment itself has even been observed to influence teratoma formation of transplanted hES-derived neural progenitor grafts following MCAO in rats (Seminatore, Polentes et al. 2010). ES cell grafts also carry the possibility of immune rejection by the recipient. Possibly strategies to minimize immune rejection for ES cell grafts include genetic manipulations of cells to reduce potential for rejection and banking of cells with defined major histocompatibility complex (MHC) backgrounds (Thomson, Itskovitz-Eldor et al. 1998). Although ES cells represent powerful tools for cellular therapy and despite the ethical and moral issues that abound, more studies are needed to ensure the use of ES cells can be a safe therapy for ischemia.

Induced pluripotent stem cells (iPS cells)

Researchers at Kyoto Univeristy in Japan first described induced pluripotent stem cells (iPSCs) in 2006. In a seminal paper titled "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors", Takahashi and Yamanaka demonstrated that adult fibroblasts could be "reprogrammed" to an ES cell-like state by the introduction of 4 factors: Oct3/4, Sox2, c-myc, and Klf-4 (Takahashi and Yamanaka 2006). Their groundbreaking discovery challenged convential thought about the plasticity of somatic cells (Ben-David and Benvenisty 2011). iPS cells generated in their study exhibited ES cell morphology, ES growth properties, expressed ES cell markers, and showed similar patterns of gene expression and DNA methylation (Takahashi and Yamanaka 2006). Furthermore, when transplanted into nude mice, mutipotent capacity was demonstrated because they generated tumors consisting of cells derived from all 3 germ layers (Takahashi and Yamanaka 2006). Although initial work was done in murine cells, Takahashi also demonstrated the reprogramming of adult human somatic cells with the same 4 factors (Takahashi, Tanabe et al. 2007). Work in humans is important in demonstrating the principal that human ES-like cells can be obtained without destruction of embyros. Because iPSCs do not rely on embryonic sources, iPSCs do not face the ethical, moral, and legal issues faced by ES cells. Since their discovery, there has been an explosion of research aimed at improving reprogramming techniques and implementing cells into research on animal models

of CNS pathologies (Hockemeyer, Soldner et al. 2008, Maherali, Ahfeldt et al. 2008, Okita, Nakagawa et al. 2008, Feng, Ng et al. 2009, Lin, Ambasudhan et al. 2009, Patel and Yang 2010).

Although iPSC research is still in its infancy, iPS cells have immense potential as tools for modeling human disease, drug discovery, and cell transplantation therapies (Okita 2010, Brennand, Simone et al. 2011). In addition to exhibiting ES cell-like properties, iPSCs are of particular interest for transplantion therapies to researchers and clinicians because of the potential to generate autologous, patient-specific cells. The use of autologous cells could feasibly sidestep the hurdle of immune rejection that currently faces the use of clinical ES cells because donor major histocompatibility complexes should be identical to recipient. As a proof of principal, researchers have already demonstrated that autologous iPS cells can be generated from patients with a variety of CNS pathologies, such as amyotropic lateral sclerosis (ALS) and schizophrenia (Dimos, Rodolfa et al. 2008, Brennand, Simone et al. 2011).

The use of iPSCs for ischemic stroke therapy has great potential. Studies on the transplantation of iPSCs for ischemic stroke therapy in animal models have already begun, but have exhibited mixed results thus far. To be discussed more in the following section, iPSCs not only exhibit the putative therapeutic properties of ES cells, but also share their pitfalls, such as tumor formation (Ben-David and Benvenisty 2011). Kawai and colleagues (2010) demonstrated the risks associated with the intracerebral transplantation of undifferentiated iPSCs in an MCAO rodent

model of stroke. Animals that received the transplants developed teratomas and faired worse in behavioral tests than sham animals (Kawai, Yamashita et al. 2010). Other studies have been more promising. Also in 2010, Chen et al (2010) and Suzuki et al (2010) saw functional improvements after iPS transplants. Chen et al performed sub-dural transplants with fibrin glue to increase iPS graft adhesion in rats following MCAO occlusion and Suzuki et al in a rodent model of hind-limb ischemia. While we are enthusiastic about the study of Chen et al (2010), the decision to pre-treat animals in the study with iPS grafts before the onset of ischemia raises questions about the clinical relevance of their approach (Chen, Chang et al. 2010, Suzuki, Shibata et al. 2010). More recently, a 2011 study by Jiang and colleagues used intracerebral transplantation of iPSCs reprogrammed from human fibroblasts in an MCAO model of stroke. iPSCs were injected ipsi- and contralateral to the infarct in female rats following MCAP. Groups receiving iPSC transplants showed a lower infarct volume and improved sensorimotor function compared with animals receiving non-reprogrammed primary fibroblasts (Jiang, Lv et al. 2011).

As stated, the potential to transplate iPSC therapy into clinical applications is hindered by the primary factor that also hinders ES cells: tumorigenicity (Ben-David and Benvenisty 2011). Tumorigenecity is an issue inherent in initial reprogramming methods because the 4 reprogramming factors used to reprogram cells have all been associated with tumors or are oncogenes themselves (Ben-David and Benvenisty 2011). The traditional reliance on retroviruses for reprogramming runs the additional risk of re-activation at a later point in time following

transplantation. Research into alternative reprogramming methods is ongoing, which may provide efficient methods to generate iPSCs while diminishing their tumorigenic capacity (Hockemeyer, Soldner et al. 2008, Maherali, Ahfeldt et al. 2008, Okita, Nakagawa et al. 2008, Feng, Ng et al. 2009, Lin, Ambasudhan et al. 2009, Patel and Yang 2010). Initial research on comparisons of iPSCs and ESCs suggested high levels of genetic similarities, however it is becoming clear that substantial differences in DNA methylation, copy number variation, and genetic imprinting exist between iPSCs and ESCs (Kim, Doi et al. 2010, Meissner 2010, Barrero and Izpisua Belmonte 2011, Ben-David and Benvenisty 2011, Gore, Li et al. 2011, Lister, Pelizzola et al. 2011). Some work has shown that early passage iPSCs retain an epigenetic profile similar to the donor tissue, suggesting iPSCs have the capacity to retain epigenetic "memory" (Kim, Doi et al. 2010, Sullivan, Bai et al. 2010). This may actually be beneficial for establishing *in vitro* model systems for CNS pathologies with strong epigenetic components. Regardless, more work is needed on the relationship between iPSC genetics and epigenetics and the phenotypic/functional behavior of iPSCs before they can be translated to clinical studies involving transplantation (Barrero and Izpisua Belmonte 2011).

Cellular Therapy: Timing, Delivery, Survival, and other considerations

There are many considerations that must be made if cellular therapy is to translate into clinical success. Proper dosages, source of cells, timing of delivery,

and the route of delivery are all things that need to be considered among other things (Sahota and Savitz 2011). Clinical trials to date have demonstrated as a proof of principal that cellular transplantation for stroke is clinically safe (Kondziolka, Steinberg et al. 2005, Lee, Hong et al. 2010). Cellular therapy for stroke is still in its infancy and more work is needed to optimize the approach. Learning from the pitfalls of translation from neuroprotection studies, the American Stroke Association has made early attempts to address issues and considerations that need to be taken into account in order for cellular therapy to successfully translate to the clinic. More detail on considerations for cellular therapy can be found in the Stem Cell Therapies as an Emerging Therapy for Stroke (STEPS) guideline publications (2009, Savitz, Chopp et al. 2011). We now present examples of considerations that should be taken for cellular therapy for ischemia.

Timing

As with any therapy for ischemic stroke, the timing of delivery must be considered if the desired therapeutic efficacy is to be obtained. There are several phases of stroke recovery and therefore several windows of time. Any therapy aimed at neuroprotection must be delivered acutely (within 24 hours for human patients). Cellular therapy holds promise for longer windows of time than traditional neuroprotective and thrombolytic approaches. IV or IA administration may be most beneficial within 1 month before glial scar formation is complete, a

time window which may not be suitable for intracerebral transplantation due to hostility of ischemic cellular microenvironment. Intracerebral transplantation may be best suited at longer time points after ischemia, after mechanisms of ischemic cell death have been down-regulated, and when formation of a glial scar may prevent IV or IA therapy from being beneficial (Hess and Borlongan 2008, Bliss, Andres et al. 2010).

Intracranial Delivery

Intracranial delivery is the most direct route for cellular delivery, capable of delivering the most cells to the infarct (Hess and Borlongan 2008). Clinical studies to date have demonstrated the safety of intracranial delivery methods for stroke (Kondziolka, Steinberg et al. 2005), however it is unclear if patients receive any functional benefit. As stated, the ischemic cortex is a hostile environment as a result of pro-inflammatory cytokines, oxidative stress, and glutamate-mediated excitotoxicity and transplanted cell survival is low. Intracerebral transplantation routes may be best suited after glial scar formation (about 1 month after ischemia) when the cortical environment becomes more hospitable. Furthermore, intracerebral grafts may require a physical scaffold to adhere to due to loss of tissue following ischemia (Hess and Borlongan 2008).

Intravenous Delivery

Intravenous delivery of stem cells has been studied at great length, with MSCs receiving special attention. As discussed, the exogenous stem cells can exert therapeutic benefit without replacing cells. The current body of evidence actually supports the notion that intravenous cell based therapy does not act through cell replacement, rather through modulation of neuroinflammation, growth factor production, endogenous neurogenesis, and angiogenesis (Hess and Borlongan 2008). Clinical trials of IV MSCs to date have shown that IV MSCs are safe and the transplanted cells provide some benefit (Lee, Hong et al. 2010). IV or IA administration may provide most benefit during time periods when chemoattractive chemokines are still up-regulated in order to home cells toward the infarct (Bliss, Andres et al. 2009). Caution must be taken with IV or IA delivery. Hematically delivered cells have the potential to obstruct pulmonary and arterial vasculature and, since administration is systemic, there is potential of buildup of cells in other organs that may cause detrimental side effects (Hess and Borlongan 2008, Bliss, Andres et al. 2009).

Intranasal Delivery

Intranasal administration (INA) of cells has recently surfaced as a novel method for cellular delivery to the CNS that sidesteps the blood-brain barrier (BBB).

Intranasal delivery of peptides and other therapeutic agents has previously shown therapeutic benefit for several diseases in animal models and in humans (Henkin 2011). Danielyan and colleagues first demonstrated that cells could be administered into the CNS through INA (Danielyan, Schäfer et al. 2009). Since then, intranasal delivery of MSCs has shown to be beneficial in rodent models of neonatal hypoxia/ischemia (van Velthoven, Kavelaars et al. 2010) and in rodent models of Parkinsons (Danielyan, Schäfer et al. 2011). The exact route taken by intranasally administered agents is not well understood, however it is thought that the RMS plays an important role (Scranton, Fletcher et al. 2011). INA seems to be a promising novel route for cellular delivery, however more studies examining the precise mechanism of intranasal delivery are needed.

Cell Survival and Tumorigenesis

The ischemic cortex is a hostile environment and as a result, survival of exogenous and endogenous cells at the ischemic border is often low. Future studies examining methods to increase cell survival in the ischemic cortex would be of great benefit to the cellular therapy field. Genetic modifications and hypoxic preconditioning (both discussed in the following sections) are two such methods that have sought to improve cell survival after transplantation. Proper dosage studies may be needed in order to determine the optimal number of cells to administer that provides the greatest benefit while minimizing the risk of

tumorigenesis. Additionally, the transplantation of multipotent cells carries an inherent risk of tumorigenesis, and longitudinal studies are needed across all cell types and routes of administration to ensure cellular therapy does not induce tumorigenesis following administration.

Summary: Cellular Therapy and Its Implications

Cellular therapy, the use of stem cells for treatment, is an exciting new avenue for stroke with many possibilities. Thanks to studies dating back to the 1960's, it is now known that the central nervous system does indeed produce new neurons in some areas of the brain and that these cells are involved in endogenous repair of the CNS after injury. Although this is important and exciting, this endogenous repair response does not seem to occur to a large enough degree to have broad implications for CNS repair and patient recovery. Therapies that bolster this endogenous response, either by increasing proliferation of these cells or by increasing their migration to the stroke injury, may be fruitful and benefit stroke patients in the future.

Transplantation of exogenous cells shows even more promise. Much research has been done in the past two decades demonstrating possibilities that were once thought to be simply science fiction. Stem cells from a variety of sources can be transplanted in the brain to supplement the injured tissue with growth factors, and in some cases cells can be transplanted that will terminally differentiate into neurons and glia, opening up the possibility for replacement of dead cells.

Cellular therapy has opened up many exciting avenues for novel stroke therapies. Current research shows the possibilities cellular therapy has to offer; however in its current form, cellular therapy may not be ideal for promoting widespread repair and recovery. Concerns such as low graft survival, immune rejection, and tumorigenesis exist in the lab and may preclude the success of cellular therapy for stroke. Additional strategies to improve upon current cellular therapies may be necessary for successful future clinical implementation.

Chapter 3 - Strategies to Improve Cellular Therapy for Ischemic Stroke and Focal Adhesion Kinase (FAK)

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Deveau TC, Yu SP, Wei L. "Cellular Therapy for Ischemic Stroke" Translational Stroke Research: From Target Selection to Clinical Trials (Springer Series in Translational Stroke Research) by Paul A. Lapchak and John H. Zhang. Springer Publishing March 23, 2012. pp. 777-817. DOI : 10.1007/978-1-4419-9530-8.

Cellular Therapy: Modifications and Looking Toward the Future

Studies to date utilizing cellular therapy for ischemia have laid the foundation as a proof of principal and demonstrated beneficial effects of exogenous cell transplantation and enhancement of innate neurogenesis. As stated, the cellular microenvironment of the peri-infarct is a hostile environment for cells, and as a result, cell survival is low and perhaps not reaching its full potential. Some early studies undertaking optimization of cellular therapy have been undertaken already that show promise in improving cellular therapy *in vitro* and in animal models. We now present some early evidence that suggests optimization of cellular therapy and combinatorial therapy may be beneficial for cellular therapy. Because ischemic stoke is a heterogeneous insult, treatment strategies that provide combinations of pharmacology, gene therapy, cellular therapy, and rehabilitation may be needed for future translational success.

Genetic Modifications: Over-expression

Studies involving genetically modified stem cells and ischemia go back to at least the mid-2000s. Several groups have shown that stem cells over expressing genes of interest improve outcome measures over non-modified cells in several animal models of disease. Genetic modification of stem cells may be beneficial in improving survival and homing to sites of ischemic injury. Additionally, genetically modified cells have the potential to serve as a drug delivery vectors, eliminating the need for additional drug adminstration.

Wei and colleagues tested the hypothesis that genetic modification of transplanted cells may enhance recovery after stroke. Mouse ES cells were modified to over express the antiapoptotic gene BCL-2 and transplanted into the ischemic hemishphere of rats 7 days after transient MCAO. Cell survival and neuronal differentiation of modified mES cells was increased over normal mES cells, and animals who received modified mES cells showed greater long-term functional recovery 21 and 35 days after transplantion compared to animals who received normal mES cells (Wei, Cui et al. 2005). Ikeda et al transplaned rat BM-MSCs overexpressing human FGF-2 into the striatum of rats 24 hours after MCAO. They observed decreased infarct volume and improved neurological scores at days 7, 14, and 21 in the FGF-2-MSC group over non-overexpressing MSC group (Ikeda, Nonoguchi et al. 2005). More recently, Wu and colleagues transplanted NSCs from embryonic rat hippocampi that were engineered overexpress hypoxia inducible factor-1 alpha (HIF-1α) into adult rats after transient MCAO. They observed that animals who received the modified cells showed more functional improvement 7,

14, 21, and 28 days after MCAO than any other of their experimental groups (Wu, Chen et al. 2010).

Other animal models have been examined as well. There are many parallels between cellular damage in myocardial ischemia following myocardial infarction and cerebral ischemia following cerebrovascular occlusion. Cellular therapy for myocardial infarction faces difficulties in cellular survival similar to ischemic stroke. Using MSCs over-expressing the anti-apoptotic gene BCL-2, Li and colleagues demonstrated the benefit of cells overexpressing BCL-2. They saw that transplantation of BCL-2 MSCs into the myocardium decreased the number of TUNEL positive cells, increased VEGF secretion, increased vessel density, and decreased scar size compared to vector MSCs in a rodent model of myocardial infarction (Li, Ma et al. 2007). Benefit has also been seen in models of spinal cord injury. Transplantation of hNSCs over-expressing anti-apoptotic BCL-X_L in female rats 7 days after spinal cord injury increased graft survival, increased white matter volume, decreased cavity volume, and improved functional recovery compared to transplanted normal hNSCs (Lee, Kim et al. 2009).

Research in animal models to date suggests that genetically modifying cells to over-express proteins may be beneficial for not only graft survival but for functional recovery. Only a handful of candidate proteins have been tested, leaving countless others to be investigated. Future studies should also examine the effect of protein over-expression in different cell types. The effects of over-expression of a certain gene of interest may not carry over across various cell types. Last, care must

be taken with cells over-expressing proteins such as growth factors, which are normally strictly temporally and spatially regulated endogenously. Long-term longitudinal studies are needed for transplantation studies using cells over-expressing such genes of interest to demonstrate the safety of this approach.

Hypoxic preconditioning

Ischemia induces a hostile environment in cortical tissues. Not only do endogenous cells die as a result of necrosis and apoptosis, the microenvironment in the ischemic penumbra is hostile to migrating endogenous NPCs and exogenous cellular grafts. As a result, the survival of migrating and transplanted cells is often very low. By utilizing the innate oxygen sensing system of the cell, preconditioning cells with a sub-lethal hypoxic insult can be protective and even increase therapeutic efficacy after transplant.

Hu et al (2008) demonstrated that hypoxic preconditioning of murine BM-MSCs provided benefits over normoxic BM-MSCs in a rodent model of myocardial infarction. HP-BM-MSCs had a greater rate of survival, smaller infarct size, and greater cardiac vessel density after transplantation than normoxic BM-MSCs (Hu, Yu et al. 2008). *In vitro* studies using human embryonic cells have shown that, over control, hypoxic preconditioning increases neural differentiation, action potential amplitude and number of APs, cell survival following ischemic insult, and delayed up-regulation of erythropoietin, VEGF, and the pro-surival gene BCL-2 (Francis and

Wei 2010). The benefits of hypoxic preconditioning for stem cells has been demonstrated in other animal models of ischemia, and have been shown to benefit ischemic tissue re-growth in rodent hindlimb ischemia models (Leroux, Descamps et al. 2010). Hypoxic preconditioning also has demonstrable benefits for other stem cell types in animal models of ischemia, such as ES cells (Theus, Wei et al. 2008)

Hypoxic preconditioning represents a promising method to improve exogenous graft survival. While hypoxic preconditioning undoubtedly involves activation of the cellular HIF system, further studies should investigate the mechanistic interplay between hypoxic-preconditioned grafts, host tissue, and cellular survival.

Combinatorial Therapies: Cellular Therapy and Environmental Enrichment

As previously stated, successful clinical therapies for stroke will most likely encompass multiple mechanisms of action to provide neuroprotection and promote CNS recovery. Additionally, stroke is a debilitating injury that will most likely rely, not only on acute clinical treatment, but also long-term rehabilitation to promote a full recovery and significantly improve the quality of life for patients. Successful stroke therapy will not only involve multiple mechanisms of action on the cellular level, but combine clinical care with another modality such as rehabilitation or sensory stimulation to promote systems-level recovery.

Previous studies have examined the effect of environmental enrichment and rehabilitation in animal models of ischemia. Environmental enrichment can enhance the intrinsic self-repair of the CNS following ischemia. Environmental enrichment is seen to increase dendritic arborization (Johansson and Belichenko 2002), endogenous neurogenesis (Komitova, Mattsson et al. 2005), and growth factor production (Ickes, Pham et al. 2000, Hicks, Lappalainen et al. 2009). Hicks and colleagues have demonstrated the beneficial effects of environmental enrichment and adult murine NPC transplantation following ischemia in rats (Hicks, Hewlett et al. 2007) and the trend of environmental enrichment on increasing human ES cell survival following transplantation after ischemia in rats (Hicks, Lappalainen et al. 2009).

Clinically, many patients undergo some sort of rehabilitation therapy following stroke. It therefore seems prudent to examine the effect of environmental enrichment and rehabilitation combined with cellular therapy. Initial studies on environmental enrichment and cellular therapy have shown potential. Future studies should examine the effect of environmental enrichment and different task/context-specific rehabilitation paradigms combined with cellular therapy on CNS recovery.

Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a 125kD non-receptor protein kinase first identified in 1992 as a substrate of the viral Src oncogene (Burgaya and Girault 1996, Mitra, Hanson et al.). FAK is expressed ubiquitously throughout the body and at high levels throughout the CNS (Burgaya, Menegon et al. 1995, Burgaya and Girault 1996, Contestabile, Bonanomi et al. 2003). FAK knockout is embryonic lethal, demonstrating the importance of FAK in normal development (Xiong and Mei 2003). FAK is highly important in cellular migration and thought to serve as a cellular integrator between cell-ECM interactions, growth factor signaling, and cytoskeletal dynamics (Burgaya and Girault 1996, Mitra, Hanson et al. 2005). FAK has also been related to adhesion dependent apoptosis, cell survival (Westhoff, Serrels et al. 2004), and cell cycle progression (Lim, Chen et al. 2008). In neurons, FAK plays an essential role in neuronal migration during development (Xie, Sanada et al. 2003, Li, Lee et al. 2004, Nikolic 2004, Kawauchi and Hoshino 2008), axon growth (Robles and Gomez 2006), synapse remodeling (Rico, Beggs et al. 2004), and LTP induction (Burgaya and Girault 1996, Xiong and Mei 2003, Yang, Ma et al. 2003). Additionally, we have previously reported on the role of FAK in cellular migration, wound healing, and interaction with neuronal ion channels (Wei, Wei et al. 2008). FAK is related to many aspects of cellular mechanics and improvement upon the role of FAK in cellular mechanics would be benefit stem cells following transplantation into ischemic brains.

Protein Structure and Activation

Focal Adhesion Kinase (FAK, pp125^{FAK}, PTK2) is a highly conserved 125kd non-receptor protein kinase first identified in transformed chicken embryo cells as a substrate of v-Src. It has over 90% sequence homology across species (including human and mouse) and has been mapped to human chromosome 8 and mouse chromosome 8 (Zhao and Guan 2011). FAK is a primarily a cytosolic protein and acts as a "signaling switch" between cells and the extra-cellular matric (ECM) at focal adhesions and a signaling scaffold. Structurally, the FAK protein consists of 3 main domains (from N-terminus to C-terminus): FERM (4.1, ezrin, radixin, moesin) domain, catalytic kinase domain, and FAT (focal adhesion targeting) domain (Lietha, Cai et al. 2007). FAK is normally autoinhibited by the binding of the FERM domain to the kinase domain. The crystal structure of autoinhibited FAK and activated FAK was recently solved (Lietha, Cai et al. 2007). Upon activation, Tyr 397 is autophosphorylated, FERM inhibition removed, Src binds to FAK and phosphorylates Tyr 576 and 577, and the FAK/Src complex phosphorylates downstream targets (Zhao and Guan 2011). The structure of the FAK protein is illustrated below in **Figure 3.1**.

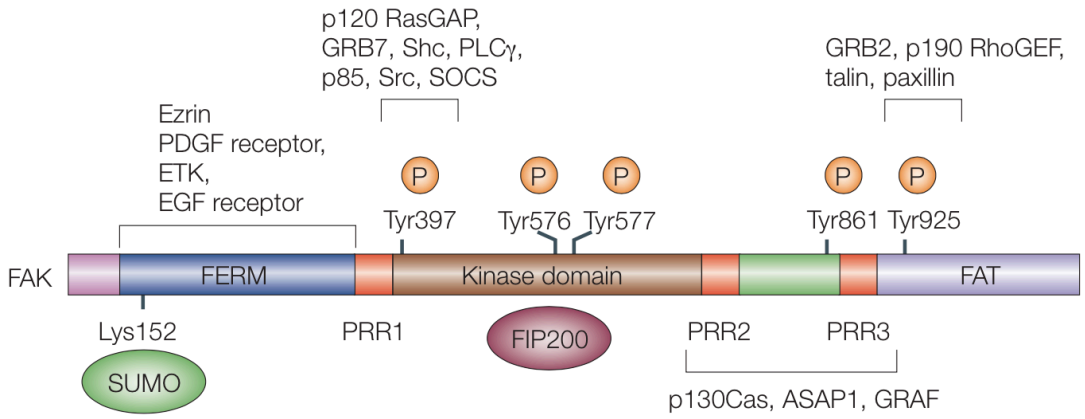


Figure 3.1. Focal Adhesion Kinase Domain Structure and Phosphorylation Sites.

Focal adhesion kinase (FAK) contains a FERM (protein 4.1, ezrin, radixin and moesin homology) domain, a kinase domain and a focal adhesion targeting (FAT) domain. The FERM domain mediates interactions of FAK with the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, the ETK tyrosine kinase and ezrin, and the FERM domain can be conjugated to SUMO (small ubiquitin-related modifier) at Lys152. The FAT domain recruits FAK to focal contacts by associating with integrin-associated proteins such as talin and paxillin. It also links FAK to the activation of Rho GTPases by binding to guanine nucleotide-exchange factors (GEFs) such as p190 RhoGEF. FAK contains three proline-rich regions (PRR1-3), which bind Src-homology-3 (SH3) domain-containing proteins such as p130Cas, the GTPas regulator associated with FAK (GRAF) and the Arf-GTPase-activating protein ASAP1. FAK is phosphorylated (P) on several tyrosine residues, including Tyr397, 407, 576, 577, 861 and 925. Tyrosine phosphorylation on Tyr397 creates a Src-homology-2 (SH2) binding site for Src, phospholipase Cγ (PLCγ), suppressor of cytokine signaling (SOCS), growth-factor-receptor-bound

protein 7 (GRB7), the Shc adaptor protein, p120 RasGAP and the p85 subunit of phosphatidylinositol 3-kinase (PI3K). Phosphorylation of Tyr576 and Tyr577 within the kinase domain is required for maximal FAK catalytic activity, whereas the binding of FAK-family interacting protein of 200kDa (FIP200) to the kinase region inhibits FAK catalytic activity. FAK phosphorylation at Tyr925 creates a binding site for GRB2. Figure and text adapted with permission from Macmillian Publishers Ltd on behalf of Nature Publishing Group: Figure 2, Mitra SK et al. Focal Adhesion Kinase: In Command and Control of Cell Motility. *Nature Reviews Molecular Cell Biology* **6**, 56-68 (January 2005) | doi:10.1038/nrm1549. Copyright 2005.

The N-terminus of FAK is comprised of a FERM domain. FERM domains are present in a variety of mammalian proteins and it is implied that the function of these domains is to link the cell with the cytoskeleton at specific cellular locations. Although predominantly located in the cytoplasm at focal adhesion sites, the tri-lobed FERM domain of FAK has a nuclear localization sequence (NLS) as well as a nuclear export sequence, and nuclear functions of FAK have been observed as well (Lim, Chen et al. 2008, Lim, Chen et al. 2008, Frame, Patel et al. 2010, Lim 2013). Most importantly, the FERM domain of FAK is involved in FAK auto-inhibition. At resting state, FAK is normally inactive. The FERM domain is responsible for autoinhibition of the kinase domain of FAK through binding of the F1 FERM lobe to a linker segment containing Y397 and a pocket within the F2 lobe binding to F596, a reside within the FAK kinase domain (Lietha, Cai et al. 2007, Zhao and Guan 2011). The FERM domain is also associated with a number of other proteins, including ETK, Ezrin, the PDGF receptor, and the EGF recptor. Sumolation of this domain can occur as well, and is associated with nuclear translocation (Mitra, Hanson et al. 2005).

FAK next contains a catalytic kinase domain. As stated, FAK is a non-receptor protein kinase that is normally autoinhibited by binding of the FERM domain to the N- and C-terminal lobes of the kinase domain. FAK is activated by integrin or growth factor activation, which leads to autophosphorylation on Tyr 397. The mechanism of Tyr 397 autophosphorylation is currently unclear. FAK activation and Tyr 397 phosphorylation induces a conformational shift that allows for Src and

Src family kinase recruitment and subsequent binding between FAK and Src. Src binding further activates FAK by phosphorylating FAK at Tyr positions 576 and 577. The activated FAK/Src complex then phosphorylates additional tyrosine residues on FAK, and recruits and activates downstream subtrates including paxillin, p130CAS, and effects Rho-family GTPase-mediated cytoskeletal reorganization (Lietha, Cai et al. 2007, Frame, Patel et al. 2010, Zhao and Guan 2011).

The C-terminus of FAK contains a FAT (focal adhesion targeting) domain as well as proline-rich areas that serve as binding sites for a variety of other proteins with SH3 domains, such as p130CAS (Girault, Costa et al. 1999). The FAT domain of FAK is linked to the catalytic domain with a linker that includes a proline-rich low complexity region (Lietha, Cai et al. 2007). The FAT sequence is "necessary and sufficient" for localization of FAK to focal adhesions and FAK C-terminus is thought to determine its subcellular localization (Hildebrand, Schaller et al. 1993, Burgaya and Girault 1996). This domain recruits FAK to focal adhesions through binding of proteins such as talin and paxillin. This domain is also responsible for the linkage of FAK to the Rho-family GTPases. Phosphorylation of Y925 additionally creates a binding site for GRB2 (Mitra, Hanson et al. 2005).

FAK and Cellular Adhesion

Attachment and adhesion are very important aspects of any sort of exogenous cell transplantation for ischemia. Transplanted cells require proper

ECM/cell attachment at their destination for survival. Without such, transplanted cells risk undergoing anoikis, which is apoptosis caused by improper ECM/cell attachment (Gilmore 2005). Integrin-mediated interactions between the ECM and the cell not only serve to anchor the cell, but activate intracellular signaling cascades crucial for cell survival. ECM/cell signaling and attachment is especially relevant when considering treatments for ischemic stroke. Stroke causes blood-brain barrier disruption and ECM disregulation through mechanisms such as matrix metalloproteinase up-regulation (Lipton 1999). Transplanted cells that reach the ischemic brain must settle into an area with a disrupted ECM. Cellular mechanisms that improve upon ECM/cell attachment in this context would be beneficial for transplanted stem cells.

FAK is a crucial integrator of signaling between integrins in the ECM and cell and the cytoplasm of the cell. FAK is therefore in a unique position to modulate interactions between the host ECM and transplanted cells. The FERM domain on the N-terminus of the FAK protein interacts with the cytoplasmic β tails of integrins. Integrin stimulation then activates FAK, leading to Src binding, and activation of downstream signaling cascades involving cellular dynamics and mechanics (Frame, Patel et al. 2010). Several studies to date have examined the affect of FAK on anoikis. One study has shown by inhibiting FAK in cultured fibroblasts through peptide inhibition, apoptosis resulted in a dramatic percentage of cells as detected with apoptosis labeling kits (Hungerford, Compton et al. 1996). Another study demonstrates in cultured epithelial and endothelial cells that express constitutively

active forms of FAK show less apoptosis than their control counterparts following detachment as measured by DNA fragmentation (Frisch, Vuori et al. 1996).

Cells that are transplanted into the ischemic brain face a hostile environment upon transplantation. Establishing successful connections between the cells and a disrupted ECM is one of the major hurdles that faces cell transplantation of all types. FAK plays a vital role in cell survival signaling when it comes to interactions between the ECM and the cell. Increasing FAK activity in transplanted cells may provide a mechanism through which they are better able to cope with and adjust to their new environment and settle into the host ECM.

FAK and Cellular Migration

Cell migration is a generic term that refers to the translocation of a cell from one location to another. More specifically, cell migration is a process of highly coordinated and integrated events involving cell polarization, protusion (lamellipodia and filapodia) formation and adhesion, cytoskeletal remodeling, focal adhesion turnover, translocation of the cell, and rear retraction and release of the trailing edge (Ridley, Schwartz et al. 2003, Wozniak, Modzelewska et al. 2004). Cell migration is essential for normal development and can be the underlying cause of disease pathologies (Ridley, Schwartz et al. 2003). Relating specifically to the brain, migration of neurons in a spatially and temporally coordinated process into the appropriate cortical region and layer during development is necessary for normal

brain development. The importance of cellular migration extends to cell transplantation for stroke as well. From the local homing of cells into the infarct with intracerebral transplantation to the long distance homing and migration required by intravascular and intranasal transplantation, cellular migration is an important aspect of cell transplantation for stroke. In order to successfully integrate into host circuitry and exert the optimal therapeutic effect, cells must migrate into the proper physical position. Increasing the migratory capabilities of cells before transplant would aid this process.

Many studies to date show that FAK is highly important in cellular migration. FAK is crucial in serving as an integrator between ECM and cellular signaling, and is essential in focal adhesion turnover and cytoskeletal remodeling that occurs during cell polarization and migration in response to extracellular sensory cues. FAK expression in FAK-null cells enhances spreading and migration (Owen, Ruest et al. 1999) and rescues migration related defects (Sieg, Hauck et al. 1999). FAK activation is important in cell polarization and enhanced motility (Reiske, Kao et al. 1999, Wei, Wei et al. 2008). FAK activation is involved in neuronal migration and development *in vivo* (Desmond, Knepper et al. 2014, Wang, Song et al. 2015). Additionally, enhanced FAK activity has been shown to improve migration of embryonic and mesenchymal stem cells as well (Wang, Park et al. 2000, Lee, Lee et al. 2010, Lee, Lee et al. 2010, Yun, Ryu et al. 2010, Hu, Wei et al. 2011).

Previous studies demonstrate that enhanced FAK signaling in stem cells can contribute to enhanced migration and motility. We have previously demonstrated

that hypoxia preconditioning of MSCs increases FAK phosphorylation and specific homing of MSCs to the infarct region (Wei, Yu et al. 2013). Further studies on additional cell types are warranted.

FAK and Ischemia

When looking at potential candidates for genetic modification of stem cells for transplant, a relationship between the gene product of interest and hypoxia/ischemia (H/I) is a very important factor. Cells will be transplanted after ischemic stroke into an area that is affected by ischemia, and will most likely be subject to some form of hypoxia during preparation for transplantation and during the transplantation procedure itself. Depending on the timing of transplantation, hypoxia/ischemia may affect cells that are transplanted.

Previous literature indicates a strong role between H/I and FAK. Studies show H/I can reduce levels of FAK and FAK^{Y397P} in the brain and disrupt signaling (Zalewska, Makarewicz et al. 2005). Studies involving FAK and cardiac ischemia/reperfusion injury sheds light on a link between FAK and H/I injury. Hakim et al. have demonstrated that FAK-/- cardiomyocytes exhibit significantly reduced viability following H/I (Hakim, DiMichele et al. 2009). The same study also demonstrated that conditional ventricular FAK knockouts showed greatly increased infarct volumes and TUNEL positive cells *in vivo*. Conversely, mice with genetically elevated levels of activated FAK showed significantly reduced levels of cardio-

protection in models of ischemia/reperfusion compared to control. Both reductions in infarct volume and TUNEL-positive cells were apparent (Cheng, DiMichele et al. 2012). Literature involving FAK and cardiac H/I points mechanistically to a link between FAK and NF-κβ signaling.

Studies such as these highlight the importance of FAK signaling in H/I. Improving the levels or activity of FAK in stem cells before transplantation may aid the ability of the cells to cope with an H/I insult before, during, or after transplantation.

FAK and Neuritogenesis

From a perspective of cell replacement, the ability of transplanted cells to extend processes and integrate with host circuitry is paramount. Transplanted cells must extend dendrites to receive signals from host cells and extend axons that synapse on existing cells in order to effectively integrate and communicate with host circuitry. Existing studies have demonstrated that iPS cells have the ability to differentiate into neurons and integrate into host circuitry *in vivo* after stroke (Tornero, Wattananit et al. 2013, Chau, Deveau et al. 2014). Modifications of transplanted cells that improve neuritogenesis may enhance integration *in vivo* and warrant investigation.

In addition to the aforementioned involvement of FAK in adhesion, migration, and ischemia, FAK plays an important role in neurite outgrowth.

Developing axons contain growth cones at their distal end that respond to sensory cues in the ECM at point contacts. Netrin-1 signaling is crucial for growth cone mechanotransduction that regulates axonal pathfinding and guidance. FAK signaling at point contacts between the ECM and the growth cone has been shown to have a crucial role in the guidance of growth cones in response to Netrin-1. Previous studies demonstrate that Netrin-1 attracts axons through FAK-dependent mechanotransduction, and FAK inhibition can illicit growth cone collapse (Li, Lee et al. 2004, Ren, Ming et al. 2004, Myers and Gomez 2011, Moore, Zhang et al. 2012, Myers, Robles et al. 2012).

iPS-derived neural progenitor cells have growth cones at the distal end of growing axons that respond to chemo-attractive and chemo-repulsive sensory cues. Enhanced FAK signaling within growth cones may improve axonal pathfinding and guidance, enhancing the ability of transplanted cells to connect and integrate with host circuitry.

Summary: Increased FAK Signaling May Improve Cell Transplantation

Current studies to date demonstrate as proof-of-principal that neural progenitors can be transplanted into an ischemic brain, differentiate into neurons, and integrate into the host circuitry. While these findings lay an important foundation, cell transplantation in its current state is not ideal and optimization may be required for translation into the clinic. Because of the complex nature of cell

transplantation and the integration of new neurons into existing host circuitry, successful optimization of cells for transplantation following stroke will most likely require several cellular enhancements. For cell replacement, transplanted neural progenitor cells must survive transplantation into host tissue, migrate to the injured area, extend processes, and establish connections with existing neurons, glia, and vascular endothelial cells. As demonstrated in the previous section, FAK plays an important roll as a cellular integrator and is involved in a number of aspects of cellular function. FAK is therefore uniquely positioned to exert a multitude of effects on cell adhesion, migration, survival, and neuronal process outgrowth. Enhancements of these aspects of cell function through improved FAK signaling may be beneficial for transplantation and maximize therapeutic benefit of grafted cells.

Chapter 4 - Rationale, Aims, and Experimental Methods

Rationale and Significance

Stroke is currently the 5th leading cause of death in the United States and a major cause of disability. Current treatments options for ischemic stroke focus on reperfusion, which helps prevent additional tissue damage or patient death. tPA was a landmark in terms of FDA approved therapies and aids in reperfusion, but only a small percentage of patients are qualified to receive it and an even smaller number of patients actually receive it. tPA carries a risk of hemorrhagic transformation of ischemic stroke and its use is very conservative as a result.

There are currently no FDA-approved therapies to promote CNS repair and recovery. Stroke patients are most often left with disabilities as a result and require extensive rehabilitation and assisted care. This results in a tremendous burden of care for stroke patients, financial and otherwise. There have been extensive efforts in the past few decades to find new therapies for stroke. Most of these therapies have focused on neuroprotection involving small molecules. Although many compounds have shown promise at the bench, they have all failed to translate to successful clinical therapies. Although it is difficult to pinpoint the source of failure, it is clear that moving forward more innovative approaches are required. The infarct caused by ischemic stroke is a complex injury, involving many different pathophysiological components at the cellular and molecular level. Stroke research moving forward should ideally aim for therapies that act on multiple levels. Therapies moving forward should not only provide a degree of neuroprotection

from excitotoxicity, ROS, inflammation, and immune cell invasion but also: 1) provide growth factors for repair of damaged tissue and promotion of endogenous neurogenesis and angiogenesis and 2) provide a source of cells to replace dead neurons and glia that are lost.

Stem cells are an excellent candidate for the "ideal" stroke therapy. Depending on the timing of transplantation, stem cells can provide neuroprotection, secrete growth factors that aide in host tissue repair, modulate the host immune reaction, promote endogenous neurogenesis and angiogenesis, and they can be efficiently differentiated into neural progenitors that serve as a source of cells to replace dead cells. Although this sounds like a silver therapeutic bullet that is too good to be true, preliminary experiments in rodent models of stroke demonstrate the efficacy of stem cell therapies. Preliminary rodents studies are very exciting, however caution must be used when interpreting results. There are still many questions currently raised by stem cell transplantation for stroke. There are still issues with stem cell therapy in its current form. Death of transplanted cells, inefficient delivery of cells to the injured area, tumor formation, immune rejection, and transplantation side effects, such as damaging healthy tissue for intracerebral transplantation and the risk of neurosurgical infection, are some of the issues that currently need to be addressed if stem cell therapy will a) make it into the clinic and b) provide patients the type of recovery that is promised. Optimization of stem cell therapy is required in order to close the gap between the science fiction of stem cell transplantation and clinical reality of successful therapy.

The source of stem cells is a major consideration for successful stroke therapy. Many preclinical studies have shown benefits of MSC transplantation for stroke through a variety of transplantation routes. MSCs seem to be excellent delivery vectors for growth factors, can provide neuroprotection, and promote endogenous neurogenesis and angiogenesis. MSCs are multipotent, and their ability to differentiate into neural progenitor cells and survive in the brain after transplantation is controversial. NSCs have shown potential as well, but require invasive donation from healthy tissue and show limited proliferation after harvest. Pluripotent ES cells have demonstrated an excellent ability to differentiate into neural precursors and mature neurons, and have the paracrine effects of MSCs, however the use of ES cells is controversial due to moral and ethical concerns. These concerns limit their translation to the clinic. The advent of iPS cells has helped address many of the concerns ES cells present. iPS cells seem to have all of the desirable qualities of ES cells. The can be propagated in a pluripotent state indefinitely *in vitro*, can be efficiently differentiated into neural precursors that form functional neurons *in vitro* and *in vivo*, are created from somatic fibroblasts that can be obtained ostensibly with patient consent, and lack the moral and ethical concerns raised by ES cells. Another major strength of iPS cells is that they can be patient specific. They have immense potential for personalized medicine because fibroblasts can be obtained from a patient in the clinic, reprogrammed to form iPS cells, and then differentiated and transplanted back into the same patient. Using autologous cells in theory would assure the cells would be free of immunorejection as well.

There is great excitement for the use of iPS cells for the treatment of ischemic stroke, though there are still several hurdles that may prevent clinical success. Survival of transplanted cells is low, migration of transplanted cells is not ideal, and the degree to which integration of transplanted iPS-derived neural progenitors into host circuitry occurs is unknown. Although it is unknown, it is presumed to be low and inefficient in its current state. Several strategies exist that may benefit cell transplantation. Our lab has previously shown that overexpression of the pro-survival factor BCL-2 in ES cells improves the survival of transplanted cells and recovery in animals that receive them (Wei, Cui et al. 2005). Similar genetic modification strategies that alter intracellular pathways involved in survival, migration, and neurite outgrowth may provide additional benefit with iPS transplantation and are not only currently unexplored, but urgently warrant investigation. For reasons previously unexplained, FAK is an integrator of ECM/cell signaling at the crux of adhesion, migration, survival, and neurite outgrowth signaling pathways. It is therefore an excellent candidate for genetic modification in iPS cells for transplantation.

Transplantation routes are a very important consideration for stem cell therapies. Intracerebral transplantation is currently the most direct method, however requires invasive neurosurgery that not only damages healthy CNS tissue, but also opens the patient up to infection and other surgical complications. Intravascular transplantation shows promise, however carries a major risk of cell clumping and deposition in off-target organs, such as the lungs, liver, and kidneys. Cell clumping can lead to embolisms in these organs and cause further damage.

Intranasal transplantation is a very exciting and novel transplantation route that shows a lot of promise. A wide variety of therapeutic substances ranging from antibodies to small molecules to peptides to even stem cells themselves can access the brain through the nasal passages and can reach the infarct area. The non-invasive aspect of intranasal transplantation makes it especially attractive for CNS therapies. We have previously shown that intranasal delivery of MSCs can reach the ischemic cortex and provide functional recovery. We have additionally demonstrated that using a hypoxic preconditioning pre-transplantation *in vitro* strategy improves homing of cells to the stroke area and functional recovery through a FAK–mediated signaling pathway (Hu, Wei et al. 2011, Wei, Yu et al. 2013, Sun, Wei et al. 2015, Wei, Gu et al. 2015). Preliminary data shows that iPS-derived neural progenitor cells reach the ischemic cortex following intranasal delivery. Although this is an important observation, delivery of cells of iPS-NPCs to the ischemic cortex appears to be inefficient. FAK is expressed in these cells, and enhancements of FAK signaling through genetic means may improve delivery of cells to the infarct and therefore improve functional recovery.

This body of work is significant in establishing the first reported genetic overexpression in mouse iPS cells and the subsequent first reported iPS-NPC intranasal transplantation in a mouse model of ischemic stroke. Studies such as this that aim to improve upon current cell transplantation are important and necessary for successful clinical translation of stem cell therapies for stroke.

Specific Aims

Central Hypothesis:

Overexpression of FAK in iPS cells will improve their capacity to aid in CNS recovery and repair following ischemic stroke.

Specific Aim 1: To determine the effect of FAK overexpression on iPS-NPC migration, neurite outgrowth, and functional maturation

1.1 Create a stable iPS cell line overexpression FAK and test cell survival

1.1 Measure the effect of FAK overexpression on neuronal differentiation and functional activity of iPS-NPCs

1.1 Assess the effect of FAK oeverexpression on cell spreading, cellular migration, and neurite outgrowth of iPS-NPCs

Specific Aim 2: To examine the effect of transplantated iPS-NPCs on functional recovery in a murine model of focal ischemia

2.1 Investigate survival of iPS-NPC grafts *in vivo*

2.2 Examine iPS-NPC differentiation migration and axon outgrowth *in vivo* after transplantation in a murine model of focal ischemia

2.3 Evaluate the ability of combinatorial therapy of iPS-NPC transplantation with whisker stimulation to enhance functional recovery after stroke

We propose overexpressing FAK in iPSCs will improve cell quality for transplantation by: increasing iPSC graft adhesion, reducing apoptotic cell death, enhancing iPSC motility, and increasing capacity for neurite outgrowth in iPS-derived neurons. Improving these characteristics of cells before transplantation should improve their ability to improve functional recovery after transplantation.

Experimental Methods

MEF Preparation and Inactivation

Mouse iPS cells (WP5 line, Stemgent, Cambridge MA) were first cultured according to a two-step culture method on mouse embryonic fibroblasts (MEFS, PMEF-CFL strain, Millipore, Billerica MA). Before iPS cells were plated, MEFs were expanded for 3 passages using generic growth media consisting of DMEM (with 4.5g/L glucose, L-glutamine, and sodium pyruvate, Corning Cellgro, Manassas VA) plus 10% FBS (Atlanta Biologicals, Atlanta, GA) and 1% penicillin-streptomycin (100x, Corning Cellgro, Manassas VA) in standard tissue culture conditions. After sufficient expansion, MEFs were inactivated for two hours using mitomycin-C media consisting of DMEM, 10% FBS, and 10μg/mL mitomycin-C (Sigma-Aldrich, St. Louis,

MO). After two hours, mitomycin-C media was replaced with normal growth media and cells were allowed to recover for 24 hours. After recovery, cells were washed with 1x PBS three times, detached with 0.25% trypsin-EDTA (TE, Gibco Life Technologies, Grand Island, NY), resuspended in growth media, and plated in 6-well plates.

Mouse iPS Culture

Maintenance

Mouse pluripotent iPS cells (WP5 line, Stemgent, Cambridge, MA) were first maintained as colonies on top of a feeder layer of inactivated mouse embryonic fibroblasts (preparation described above). Pluripotent colonies were plated on top of the feeder layer with ESIM media that consisted of: DMEM (with 4.5g/L glucose, L-glutamine, and sodium pyruvate, Corning Cellgro, Manassas VA), 15% ES-qualified FBS (Gibco Life Technologies, Grand Island, NY), 1% non-essential amino acids (NEAA, 100x, Gibco Life Technologies, Grand Island, NY), 1% penicillin-streptomycin (100x, Corning Cellgro, Manassas, VA), 0.5% L-glutamine (100x, Corning Cellgro, Manassas ,VA), 0.1% β-mercaptoethanol (BME, Sigma-Aldrich, St. Louis, MO), and Leukemia Inhibitory Factor (LIF, 1:10,000, Millipore, Billerica, MA). Cells were passaged using 1x PBS, 0.25% TE, and standard passaging techniques

when colonies reached 80% confluency. ESIM media in culture wells was changed daily.

Expansion

After several passages on inactivated MEFs, mouse iPS colonies were expanded in standard T25 flasks (Corning Cellgro, Manassas, VA) pre-coated with 0.2% gelatin (prepared in ddH_20, Sigma-Aldrich, St. Louis, MO) with ESIM media. ESIM media was replenished daily. When colonies reached 80% confluency (1 day if plated at 1:4, two days if plated 1:8), cells were passaged with 0.25% TE and standard passage techniques.

Differentiation

iPS cells were differentiated using a previously described 4-/4+ retinoic acid (RA) (Bain, Ray et al. 1994, Bain, Kitchens et al. 1995, Bain, Ray et al. 1996, Kim, Habiba et al. 2009) protocol in conjunction with a novel rotary culture technique previously described by our lab (Mohamad, Yu et al. 2014). Briefly, when iPS colonies were 80% confluent on 0.2% gelatin in T25 flasks, cells were passaged using standard cell passage techniques using 1x PBS and 0.25% trypsin (T, Gibco Life Technologies, Grand Island, NY). Detached cells were resuspended in 10mL of ESIM lacking BME and LIF and transferred to 10cm tissue culture dishes. Culture dishes were placed on an oribital rotary culture shaker (The Belly Button®, Denville Scientific,

Metuchen, NJ) at 30-40 RPM under standard tissue culture conditions in a tissue culture incubator to induce embyroid body formation. ESIM media lacking BME and LIF was changed on day 2 and day 4 following the start of rotary culture. On day 4, retinoic acid (RA, all-trans, $5x10^{-7}$ M, Sigma-Aldrich, St. Louis, MO) was added to BME/LIF-lacking ESIM. ESIM without BME/LIF but with RA was replenished again on day 6 following rotary culture start.

Neurosphere Dissociation and Terminal Differentiation

On day 8 following embryoid body (day 4 after neurosphere induction), neurospheres were harvested, washed with DMEM, and enzymatically dissociated with 2mL 0.25% TE for 10 minutes at 37° C. Neurospheres in TE were agitated by hand every two minutes. At 10 minutes, TE was neutralized with the addition of 10mL ESIM. Debris was removed from the top with a pastuer pipette and cells were spun down at 1000 x g for 5 minutes. ESIM was removed, cells were resuspended in 2mL modified SATO media (Bottenstein and Sato 1979), and cells were passed through a cheesecloth filter to further dissociated neural progenitor clumps. Cells were counted using a hemocytometer and then plated in 35mm dishes coated with PDL-laminin at a density of $1.5x10^{6}$ cells in SATO media for terminal differentiation. Half changes of SATO media were performed every two days thereafter.

Viral-Mediated Stable Cell Creation

Oskar Laur at the Emory University Cloning Core created the clones mentioned below. Xinping Huang and colleagues at the Emory University Viral Vector Core carried out viral creation. The pLVCT-tTR-KRAB vector was selected as a backbone to be packaged in lentiviruses aimed at tet-inducible FAK expression [a gift from Patrick Aebischer and Didier Trono, Addgene plasmied #11642 (Szulc, Wiznerowicz et al. 2006)]. The pEGIP vector was selected as a backbone to be packaged in lentiviruses aimed at constitutive transgene expression and stable cell selection [a gift from Linzhao Cheng, Addgene plasmid #26777, (Zou, Maeder et al. 2009)]. The mCherry CDS was obtained from Addgene plasmid #20943 [a gift from Karl Deisseroth, (Zhang, Wang et al. 2007)]. The FAK CDS (NCBI REF NM_013081.1) was obtained from constructs used in a previous study performed by our lab (Wei, Wei et al. 2008). For inducible expression vectors, the FAK CDS was cloned into the pLVCT backbone creating a FAK, GFP-FAK, and FAK-GFP expression vectors. For non-inducible vectors, the GFP CDS in pEGIP was substituted for the mCherry CDS. An expression vector expressing mCherry-FAK was then created. Viruses were packaged by the Emory Viral Core using transfection and HEK293FT cells. Pluripotent iPS colonies on 2% gelatin were infected with $5x10^8$-$1.5x10^9$ IU/mL. Stable colony selection was then performed using standard stable cell selection techniques.

Non-Viral Mediated Stable Cell Creation

Oskar Laur at the Emory University Cloning Core created the clones mentioned

below. Briefly, GFP, mCherry, GFP-FAK, and mCherry FAK expression vectors were created by subcloning the GFP, mCherry. GFP-FAK, and mCherry-FAK sequences described above in place of the Nanog coding sequence in the pPy-CAG-Nanog-IP plasmid [Addgene Cambridge, MA http://www.addgene.org plasmid #13838, kind gift of Shinya Yamanaka, (Mitsui, Tokuzawa et al. 2003)]. Stable cell lines were created by transfecting the expression vectors above into pluripotent iPSCs using Lipofectamine 2000 (Life Technologies, Grand Island, NY). Forty-eight hours after transfection, puromycin (0.5–1 mg/ml, Sigma-Aldrich, St. Louis, MO) was used to select stable clones expressing GFP, mCherry, GFP-FAK, and mCherry-FAK that were pooled and maintained on MEFs. We observed transgene expression through at least 30 passages as well as after cell freezing and recovery. These cells underwent the same maintenance, differentiation, and transplantation protocol as cells without the GFP, mCherry, GFP-FAK, or mCherry-FAK markers.

Cell Attachment Assay

Following neurosphere dissociation, $3x10^5$ cells were plated in 35mm dishes coated with PDL-laminin containing SATO growth medium. 24 hours after plating, cells were washed with 1x PBS and fixed with 4% paraformaldehyde (PFA, Affymetrix, Santa Clara, CA). Cell nuclei were counterstained with Hoechst 33342 for 5 minutes (1:20,000, Life Technologies, Grand Island, NY) and dishes mounted with Vectashield (Vector Technologies, Burlingame, CA). Mounted dishes were imaged with a 10x objective on a Leica inverted microscope. The total number of attached

Hoechst positive cells in a total of 6 random 10x fields per dish was counted. Cell counting was performed using ImageJ software (NIH, Bethesda, MD).

Cell Migration Assays

Cellular migration was assayed using a trans-well (Boyden chamber) assay. Transwell inserts (8μM, BD Biosciences, San Jose, CA) were coated with Matrigel® (Corning, Tewksbury, MA) for one hour at 37°C. Care was taken to ensure there were no air bubbles and the chambers rested flat. After one hour, Matrigel® substrate was removed by vacuum pipette. 400μl SATO media with or without SDF-1α (200ng/μL, Stem Cell Technologies, Vancouver, BC) was added to wells of a transwell-receiving 24-well plate. Matrigel® coated transwell inserts were placed into the wells of the 24-well plate, and $2x10^5$ iPS-(in 100μl SATO) derived neural progenitors were plated in the inserts carefully. After 24 hours, transwell inserts were fixed with 4% PFA for 20 minutes, and non-migratory cells were scraped off the top of the insert with a q-tip. Inserts were then washed three times for five minutes each with 1x PBS, solubilized with 0.02 triton x-100 for 5 minutes, washed with PBS again, and then counterstained with phalloidin (30 minute incubation, Acti-Stain Phailloidin 488, Cytoskeleton Inc, Denver, CO) and Hoechst 33342 (5 minutes). Membranes were cut out of the transwell insert and mounted on standard microscope slides with Vectasheild (Vector Technologies, Burlingame, CA). Mounted membranes were imaged (6 random fields per membrane) and Hoescht

positive cells in the resulting images were counted using ImageJ (NIH, Bethesda, MD).

Oxygen-glucose deprivation (OGD)

For an *in vitro* ischemic cell death model, day 6 iPS-derived neurons were subjected to oxygen-glucose deprivation (OGD). To accomplish OGD, media was exchanged for a physiological buffer solution lacking glucose (120mM NaCl, 25mM Tris-HCl, 5.4mM KCl, 1.8 mM $CaCl_2$, pH to 7.4 with NaOH) and cells were incubated in a calibrated hypoxia chamber (BioSpherix) perfused with 5% CO_2 and balanced nitrogen for a final ambient oxygen level of 0.2% for 2h. The oxygen level was established, maintained and monitored by the ProOx 360 sensor (Biospherix, NY). After 2h, cells were returned to a normoxic incubator and the existing OGD media was diluted by a factor of 2 with SATO. After 24h of reperfusion and oxygenation, cell viability was assessed using a standard MTT assay. Briefly, cells were subjected to MTT reagent (Sigma-Aldrich, St. Louis, MO) for 3 hours following OGD at 37° C in standard culture conditions. The resulting purple formazan product was solubilized with detergent containing HCL overnight in a tissue culture hood. Absorbance at 570nm was read and calculated against a "no OGD" control.

Neurite Outgrowth Assays

Neurospheres were harvested and plated on PDL-laminin coated 35mm dishes with or without Netrin-1 (200μM, R&D Systems, Minneapolis, MN). Live neurospheres were imaged at 48 hours.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated from iPS-derived neurons and neural progenitor cells immediately after neurophere dissociation as well as 2-, 4-, 6-, and 8- days after harvest and plating using Trizol® (Life Technologies, Grand Island, NY). Three replicates were combined for each sample. cDNA was generated using 1μg of total RNA using a high capacity cDNA kit (Life Technologies, Grand Island, NY) and the random primers within. Target genes were amplified using standard PCR reaction and cycling conditions. PCR products were run on a 2% agarose gel, visualized and imaged with ethidium bromide (EtBR) under ultraviolet (UV) light, and bands were quantified using ImageJ software (NIH, Bethesda, MD). The following primers were used (all 5'-> 3'):

BDNF F: AGGGTTTCTTACCTGGCGAC, R: GGTGGAACATTGTGGCTTTGC

CHAT F: TCCACGCCACTTTCAGTCAG, R: ATCCTCGTTGGACGCCATTT

DBH F: TACTTTGCGGATGCCTGGAG, R: GTTGAGTCGGTCAGGCTTCA

FAK F: CAGGGTCCGACTGGAAACC, R: GTTACTTCCTCGCTGCTGGT

GAD F: ATCGCTCCACCAAGGTTCTG, R: GTGAGGGTTCCAGGTGACTG

IL6 F: GGGACTGATGCTGGTGACAA, R: CGCACTAGGTTTGCCGAGTA

IL10 F: ACTACCAAAGCCACAAGGCA, R: TGGCAACCCAAGTAACCCTTA

TH F: TCACGTCCCCAAGGTTCATT, R: GAATTGGCTCACCCTGCTTG

Immunohistochemistry

In vitro: 35mm dishes of iPS-neurons were washed once with 1x PBS and fixed for 20 minutes with 4% PFA (Affymetrix, Santa Clara, CA). Cells were washed three times with 1x PBS for 5 minutes each. Cells were then treated with 2:1 ethanol:acetic acid for 5 minutes, solubilized with 0.02% for 5 minues, and blocked with 1% fish gelatin (Sigma-Aldrich, St. Louis, MO) for an hour with PBS washes in between steps. Primary antibodies were applied overnight under a coverslip. Following primary antibody incubation, cells were washed three times with 1x PBS for 5 minutes each and then incubated with secondary antibody under coverslip for approximately one hour at room temperature. After secondary incubation, Vectashield with DAPI (Vector Technologies, Burlingame, CA) was applied, dishes were coverslipped and sealed with nail polish, and imaged.

In vivo: brains in optimal cutting temperature matrix (Sakura Finetek) were cut into 10μm coronal sections using a cryostat microstome and mounted on a coated microscope slide (8 sections/slide). Slides were dehydrated with a slide warmer at 37°C for 20 minutes, allowed to cool, and fixed with 10% formalin for 10 minutes. Slides were rehydrated in 1x PBS and treated with 2:1 ethanol:acetic acid for 5

minutes. Slides were then washed with 1xPBS three times for 5 minutes each, solubilized with 0.02% for 15 minutes, and blocked with 1% fish gelatin (Sigma-Aldrich, St. Louis, MO) for an hour with PBS washes in between steps. Primary antibodies (see **Figure 4.1** below) were applied overnight under a coverslip. Following primary antibody incubation, slides were washed three times with 1x PBS for 5 minutes each and then incubated with secondary antibody under coverslip for approximately one hour at room temperature. After secondary incubation, slides were counterstained with Hoechst 33342 for 5 minutes, washed, Vectashield (Vector Technologies, Burlingame, CA) was applied, slides were coverslipped and sealed with nail polish, and imaged.

Immunoblotting

Protein from cells and brain tissue was collected and homogenized in a modified RIPA lysis buffer (50mM Tris pH 7.8, 150mM NaCl, 0.1% SDS, 0.5% NaDeoxycholate , 1% NP40 detergent, 1mM PMSF, 1X Sigma protease inhibitor cocktail, $NaVO_4$). Homogenates were incubated on ice for 30 minutes with intermittent vortexing and centrifuged at over 14,000 x g for 15 minutes at 4°C. The soluble supernatant was transferred into prechilled tubes. Protein concentration was determined using a standard Bicinchoninic acid (BCA, Sigma-Aldrich, Sr. Louis, MO) assay. Protein was normalized and mixed with 5x SDS sample loading dye. Protein samples were run on a 10% polyacrylamide gel for approximately 4 hours and transferred to a methanol-activated PVDF membrane at 4°C overnight. After transfer, membranes

were sectioned, blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) for one hour, and washed six times for 5 minutes each with tris-buffered-saline plus tween (TBST, standard preparation). After TBST wash, primary antibodies (see **Figure 4.1** below) diluted in 5% BSA were applied, and membranes were incubated overnight at 4°C with agitation on a shaker. After primary antibody incubation, membranes were washed 6 times for 5 minutes each with TBST. Secondary antibodies diluted in 5% BSA were applied for one hour at room temperature with agitation. After secondary antibody incubation, membranes were washed three times for 5 minutes each with TBST and three times for 5 minutes each with tris-buffered-saline (TBS, standard preparation). After washing membranes were developed, scanned, digitized, and underwent densitometric quantification with ImageJ software (NIH, Bethesda, MD).

Primary Antibodies

Target Protein	Antibody	Vendor	Catalog Number	Molecular Weight	Dilution	Application
Neuronal Nuclei	NeuN	Millipore	mab377	n/a	1:100	IHC
TUJ-1	TUJ-1	Covance	mrb-435P	n/a	1:500	IHC
DCX	DCX	Santa Cruz	sc-8066	n/a	1:200	IHC
mCherry	mCherry	Abcam	ab167453	n/a	1:200	IHC
GFP	GFP	Novus	nbp1-69969	n/a	1:100	IHC
Neurofilament	SMI-312	Abcam	ab24574	n/a	1:400	IHC
FoxG1	FoxG1	Abcam	ab3394	n/a	1:200	IHC
β-actin	β-actin	Sigma	A5441	42	1:5000	WB
β-Tubulin	α-Tubulin	Cell Signalling	2146	50	1:3000	WB
Bcl-2	5OE3	Cell Signalling	2870	26	1:1000	WB
FAK	clone 4.47	Millipore	05-537	125	1:1000	WB
FAK	C20	Santa Cruz	sc-558	125	1:1000	WB
FAK	Y397P	Santa Cruz	sc-11765-R	~125	1:1000	WB
FAK	Y576P	Cell Signalling	3281S	~125	1:1000	WB
CXCR4	CXCR4	R & D	mab172	40	1:2500	WB
MMP-2	MMP-2	Millipore	19167	66/72	1:1000	WB
MMP-9	MMP-9	Millipore	13458	86/92	1:1000	WB

Figure 4.1 Primary Antibody Table

Primary antibodies used in the present study are listed above.

Electrophysiology

Whole-cell patch clamp recordings were obtained from mouse iPS cells 8-10 days after terminal neuronal differentiation using an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany) at 21–23°C. Growth media was replaced with an external recording solution consisting of: 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10mM HEPES, and 10 mM glucose at a pH of 7.4. Recording electrodes for patching were pulled from borosilicate glass pipettes (Sutter Instrument, USA) and had a tip resistance between 5 and 7 MΩ when filled with the internal solution. Internal recording solution for the electrodes contained: 120 mM KCl, 2 mM $MgCl_2$ 2, 1 mM $CaCl_2$, 2 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES at a pH of 7.2. Series resistance was compensated by 75-85%. Linear leak and residual capacitance currents were subtracted on-line using a P/6 protocol. Action potentials were recorded under current-clamp mode using Pulse software (HEKA Elektronik). Inward and outward currents were recorded under voltage-clamp mode using Pulse software (HEKA Elektronic). Data were filtered at 3 KHz and digitized at sampling rates of 20 KHz.

Stroke Induction

The Institutional Animal Care and Use Committee (IACUC) at Emory University approved all experimental and surgical procedures. Middle cerebral artery

occlusion (MCAo) was performed according to a modified version of (Wei, Rovainen et al. 1995). In brief, 8-10 week old C57BL6 mice (National Cancer Institute) were anesthetized using an intraperitoneal (IP) injection of 4% chloral hydrate (400mg/kg). Animal responsiveness was checked through pinches on the feet and base of the tail. When animals were deemed non-responsive, distal branches of the right middle cerebral artery (MCA) were permanently ligated by a 10-0 suture (Surgical Specialties Co., Reading, PA). MCA ligation was accompanied by a bilateral 0-, 4-, 7-, or 15-min ligation of the common carotid arteries (CCA). Body temperature was monitored during surgery and maintained at 37.0°C using a temperature control unit and heating pads. Animals were euthanized by decapitation following chloral hydrate anesthesia 3-, 7-, 14-, 28-, 45- days after stroke. Brains were immediately removed, mounted in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA), flash frozen on dry ice, and stored at −80 °C for further processing.

Local Cerebral Blood Flow (LCBF)

Local cerebral blood flow (LCBF) measurements were performed using laser doppler scanning as described in (Mohamad, Chen et al. 2011) with the laser tip pointing to the area supplied by the MCA. A 3x3 mm^2 area around the initial spot was scanned by the Periscan Laser Doppler perfusion imaging system and analyzed by the LDPI Win 2 software (Perimed AB, Stolkholm Sweden). LCBF was measured before stroke induction, during stroke (during MCAo), 3 days after stroke, and 7

days after stroke.

2,3,5,-triphenyltetrazolium chloride (TTC) Staining

The stroke infarct was visualized using the 2,3,5-triphenyltetrazolium chloride (TTC) staining procedure detailed in (Wei 2003). Animals were sacrified 72 hours after stroke induction. The brains were sectioned into 1-mm coronal sections using a mouse brain matrix (Harvard biosciences, South Natick, MA) and incubated in 2% (TTC) solution in a water bath at 37°C with constant agitation for 5 minutes. Sections were scanned and digitized and infarct area per section was quantified using ImageJ software (NIH, Bethesda, MD). The infarct volume (mm^3) was then determined by multiplying the area by the section thickness (1mm) and summing all the sections per animal.

Intranasal Transplantation

Intranasal administration of mouse iPS-derived neural progenitors was performed as previously described for intranasal MSC delivery (Wei, Yu et al. 2013). Briefly, 1-4 hours following ischemic stroke, animals under chloral hydrate anesthesia, were given hyaluronidase (100 U, 5μL/nostril) 30 minutes prior to cell administration. After hyaluronidase treatment, animals were given a total of $1.5x10^6$ iPS-NPCs in 100μL of SATO. Cells were administered in 5μL drops per nostril, with at least one minute between left-right nostril administration and at least two minutes between

same nostril administrations in order to reduce respiratory stress. Upon completion of cell delivery, animals were placed in an incubator to recover from anesthesia. Recovered animals were then returned to their home cages and to the animal facility where they received food and water *ad libitum*.

Adhesion Removal Test

Forelimb sensorimotor deficits after stroke were analyzed by a modified adhesive removal test (Bouet, V. et al., 2009; Freret, T. et al., 2009). Briefly, the time to contact and remove an adhesive sticker was recorded one day prior to stroke, 7 days after stroke, 14 days after stroke, and 21 days after stroke. Animals were trained with one trial per day for three days prior to testing. Animals were habituated to the testing room for 20 minutes before testing was initiated, and habituated to the test cage for 5 minutes after the initial habituation period. An adhesive sticker (Tough spots, 9.5mm diameter, cut into quarters) was applied to the padded area on the bottom of the foot of either the left or right forepaw. The mouse was then placed back into the test cage and the time to contact and time to remove the sticker was recorded with a maximum of 120s. Left and right paw order was randomized between trials. In each testing session, there were four trials (four left, four right). The first trial was considered training; the last three were averaged and analyzed for each animal. One blinded experimenter conducted all tests so the pressure of sticker application was consistent. Data was analyzed as fold change compared to before stroke for each group.

Locomotion Assessment

Locomotion assessment was performed using the TopScan System (CleverSys, Inc., Reston, VA) as previously described (Sun, Wei et al. 2015). 27-29 days after stroke, animals were individually placed in an open field apparatus and their locomotion recorded for one hour. Movement was recorded with CCD cameras. An open field set up was achieved in software by defining an internal box within the whole box. Velocity, total movement, time spent outside the small box, time spent inside the small box, and crossing over from small to large was recorded and analyzed. Animals were tested 4 at a time in 4 boxes. All animals were recorded at the same time of day for consistence.

Chapter 5 - Characterization of a Modified MCAO Stroke Model

Introduction

Reliable and reproducible animal models are necessary for the study of CNS repair and regeneration following stroke. There are several common stroke models currently employed in mice that are used to study ischemic stroke: embolic middle cerebral artery occlusion (MCAO), photothrombolic, and suture middle MCAO. Each model has strengths and disadvantages.

Embolic MCA occlusions involve injection of microbeads/macrobeads or thrombotic clot embolization. Surgeries are simple and do not require craniotomies. Beads or clots are injected into the internal carotid artery and make their way into the middle cerebral artery and vessels elsewhere in the brain. Microbead injections mimic many aspects of human stroke; however these strokes can be small and variable, making studies examining circuit repair difficult. Recent studies with microbeads show that this model can be very useful to study secondary effects of stoke, such as vascular depression (Nemeth, Shurte et al. 2012, Nemeth, Gutman et al. 2014, Nemeth, Haroon et al. 2014, Nemeth, Reddy et al. 2014). Macrobead injections lead to occlusions of the MCA similar to other MCA models described below; however these models lack reperfusion, which is an important aspect of stroke injury. Models involving blood clots can also be variable but are excellent for thrombolytic study (Carmichael 2005).

Photothrombolic stroke models utilize a photo-sensitive dye and irradiation to produce highly focal ischemic insults. Dye can be injected intraperitoneally and stroke site determined stereotaxically, so this method is simple and produces

lesions in very specific brain regions. The disadvantage of this method is that there is little ischemic penumbra, little reperfusion, and vasogenic edema that is uncharacteristic of human strokes.

Suture-mediated MCAO is perhaps the most commonly employed stroke model in animals. Most suture MCAO models involve the insertion of intraluminal filaments into the MCAO for 60-120 minutes followed by suture removal and recovery. This model is very simple; however it produces very large infarcts that span multiple brain regions (cortex, thalamas, striatum), making the study of circuit repair with these models difficult. These models are well suited to neuroprotective studies however. Poor suture placement also carries a large risk of vascular damage and hemorrhage, so care must be taken with suture placement.

We employ a modified suture-based MCAO method where 3-4 distal branches of the MCA are permanently ligated and both common carotid artery (CCA) branches are temporarily ligated. This produces a focal ischemic insult limited to the mouse sensorimotor cortex. The insult is mainly confined to the whisker barrel cortex, but extends to areas of the forepaw as well. Bloodflow during surgery drops to ~10% of normal flow and returns to ~75-80% normal levels by day 3. A distinct ischemic core and penumbra is formed and amenable to studies on neuroprotection and circuit repair. Our stroke model is illustrated in **Figure 5.1** below.

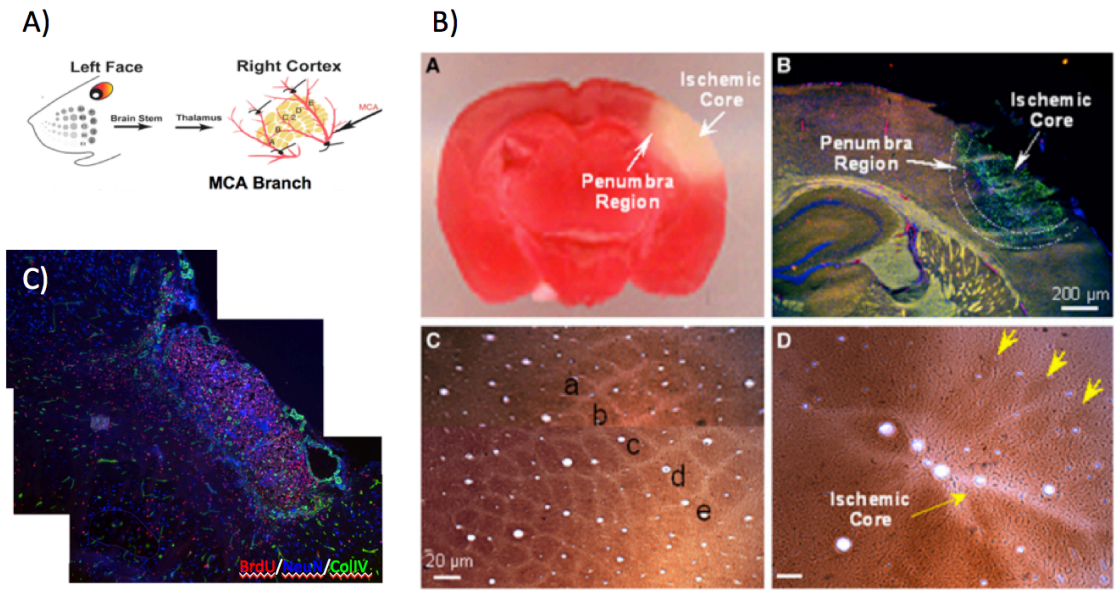


Figure 5.1. Focal Ischemic Stroke Model.

We permanently ligate distal branches of the right hemisphere middle cerebral artery (MCA, A) and temporarily occlude the bilateral common carotid artery (CCA, not pictured). An overview of the focal ischemic infarct created is presented in (B, adapted from Whitaker et al. 2007). The infarct is visualized with immunohistochemistry in (C). Figure and text adapted with permission from Macmillian Publishers Ltd on behalf of Nature Publishing Group: Figure 2, Whitaker VR et al. Whisker Stimulation Enhances Angiogenesis in the Barrel Cortex Following Focal Ischemia in Mice. *Journal of Cerebral Blood Flow & Metabolism* **26**, 57-68(May 2006). Copyright 2007.

Before beginning studies on cell transplantation, we revisited and clarified the role of common carotid occlusion in stroke induction. We assessed animals with no CCAo, 4 minute CCAo, 7 minute CCAo, and 15 minute CCAo for bloodflow, infarct volume, and cell death. Better understanding of stroke models will be beneficial for future studies of cell transplantation.

Results

Local Cerebral Blood Flow (LCBF)

Laser Doppler perfusion imaging was used to analyze the blood perfusion around the MCA territory before, during, and after surgical ischemia. Mean perfusion was analyzed in six measurements of a 3 x 3mm area overlying the occluded branch of the right MCA before, during, and 72h after stroke in each animal (Fig. 5.2). The change in blood flow was normalized to the initial baseline value for each mouse and calculated as a percentage of baseline. 4 groups of animals were measured: no CCAo, 4-minute CCAo, 7-minute CCAo, and 15-minute CCAo. Bloodflow during stroke was significantly reduced between no CCAo animals and other groups (one-way ANOVA $F(3,19)=126.8$, $p<0.0001$). Blood flow to the MCA territory was reduced by 80-90% in animals, however only reduced to ~40% in animals without CCA occlusion (**Figure 5.2**). 72 hours after stroke, bloodflow returned to 60-75% baseline in all groups. There was no difference in bloodflow between groups 72 hours after stroke.

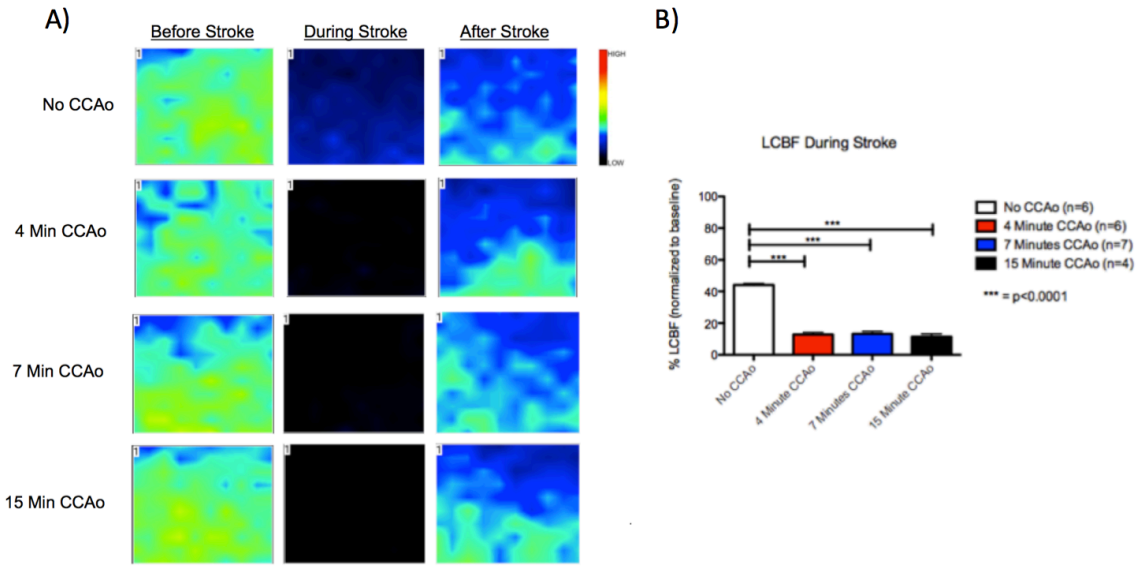


Figure 5.2. Local Cerebral Blood Flow During Ischemia.

Laser doppler scanning shows a significant reduction (A, B; one-way ANOVA $F(3,19)=126.8$, $p<0.0001$) in local cerebral blood flow (LCBF) between animals receiving permanent MCA occlusion (pMCAo) only compared to animals receiving pMCAo combined with temporary CCA occlusion (tCCAo). There was no difference between groups before stroke or 72 hrs after stroke (data not shown).

TTC Staining

Ischemic infarct volume was assessed 72h after stroke surgery. 72 hours was chosen because the bulk of cell death in the ischemic region occurs within the first 72h (Lipton, P., 1999). Beyond 72 hours, proliferative cells such as microglia and peripheral immune cells migrate into the infarct and can confounds TTC measurements. The infarct was primarily cortical and located within the vascular bed supplied by the right MCA with no detectible damage to the contra-lateral hemisphere. Indirect infarct ratios observed were as followed: no-CCAo = 0.019, 4-minute CCAo = 0.074, 7 minute CCA = 0.069, and 15-minute CCAo = 0.089 (**Figure 5.3**). There were 3-5 animals in each group. A significant difference in infarct volumes was observed between the no-CCAo group and the CCA groups (one-way ANOVA $F(3,12)=7.168$, $p=0.0051$), however there was no difference between CCA groups.

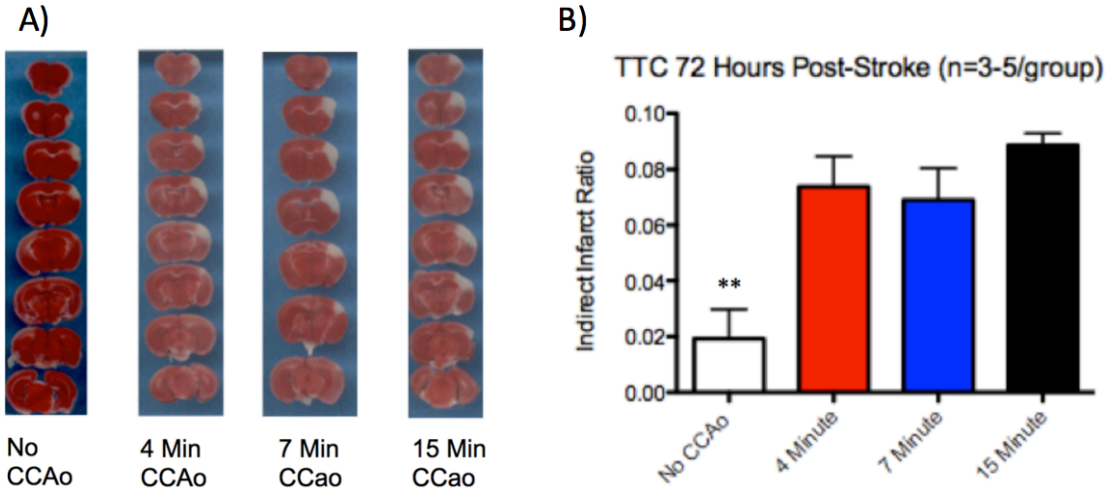


Figure 5.3. TTC Staining and Infarct Volume Assessment.

TTC staining shows decreased infarct volumes in animals receiving pMCAo only compared to animals receiving pMCAo and tCCAo (A,B; one-way ANOVA $F(3,12)=7.168$, $p=0.0051$).

TUNEL Staining

TUNEL staining was used to visualize onset of cell death. 10μm coronal sections were stained for TUNEL (green), NeuN (red), and Hoechst (blue). 6 hours after stroke induction, TUNEL positive cells started appearing in both non-CCA and 7-minute CCA groups. The NeuN signal was disrupted as well, indicative of the ischemic infarct. There were no apparent differences in the number of TUNEL positive cells, however TUNEL positive cells were localized mainly to the ventral region of the infarct in the penumbra. 12 hours after stroke induction, a large difference in TUNEL positive cells between groups can be observed. There are far more TUNEL positive cells in the 7-minute CCA group compared to the no-CCA group. Additionally, in the CCA group, TUNEL positive cells appear to be restricted to the ventral half of the infarct.

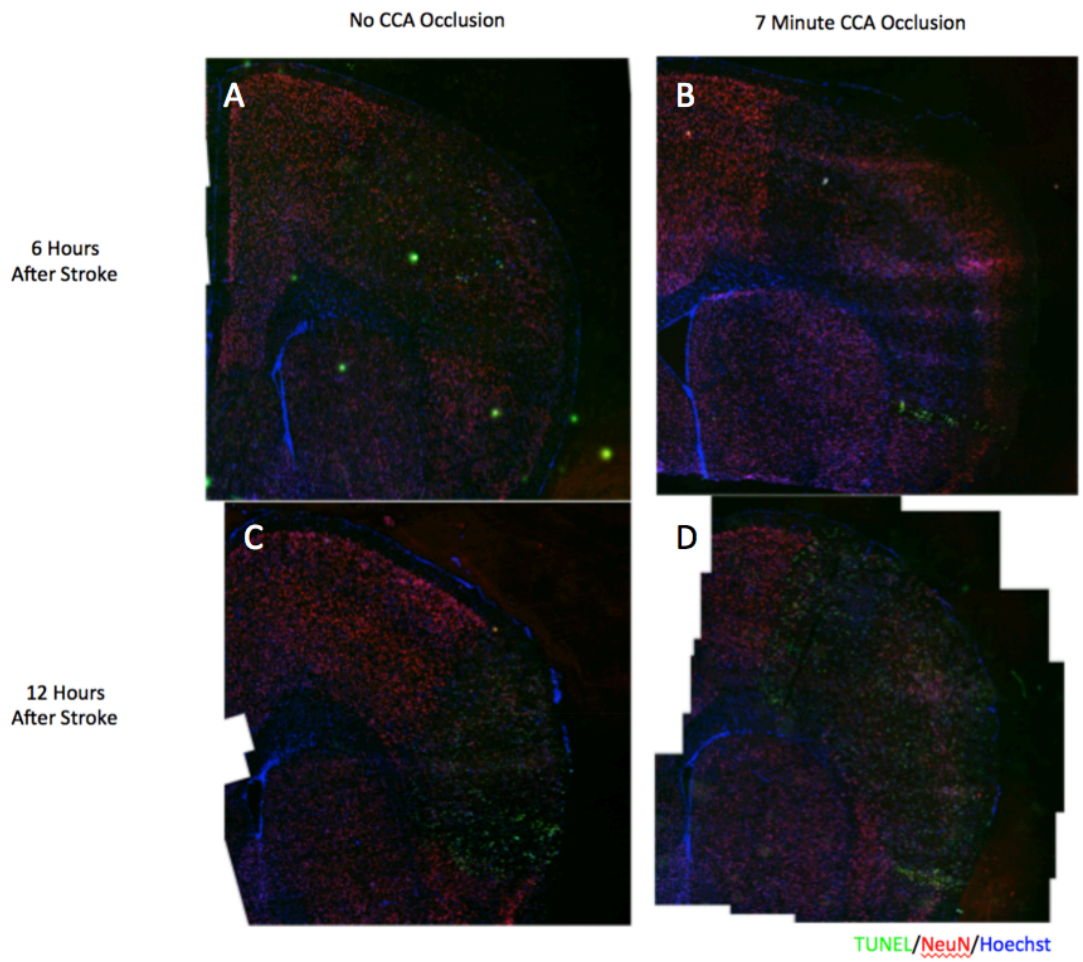


Figure 5.4. TUNEL Staining.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining shows little difference between no CCAo and 7 minute CCA0 6 hours after stroke (A, B), but demonstrates a profound difference 12 hours after stroke (C, D).

Discussion

Here we examined the effects of varying CCAo on a modified MCAo stroke model. LCBF data (**Figure 5.**2) shows a significant difference between no-CCAo and CCAo, however no differences in CCAo duration are observed. These data suggest in terms of blood flow that the effect of CCAo is "saturated" at 4 minutes and longer occlusion durations do not reduce blood flow any more in the region measured.

TTC data demonstrate the importance of CCAo in infarct formation (**Figure 5.3**). There is a significant difference again between no-CCAo and CCAo in infarct size, however no difference between CCAo groups. These data are supported by the blood flow data reported above. CCAo is important in infarct formation; however longer CCAo times do not affect blood flow during or after stroke no infarct size.

Finally, observations with TUNEL staining between no-CCA and 7-minute CCAo (**Figure 5.**4) reflect the LCBF and TTC data discussed above. There are no observable differences in the number of TUNEL positive cells 6 hours after stroke. 12 hours after stroke, however, there is a large difference in number of TUNEL positive cells. Additionally, TUNEL positive cells in the no-CCA group are restricted to the ventral half of the infarct, whereas in the 7-minute CCA group the cells are spread out throughout the infarct. This observation suggests that collateral circulation not cut off by CCA ligation may still be protecting cells in the top half of the infarct at this time point. It also suggests that cells in the dorsal half of the infarct undergo delayed cell death as a result of CCAo and may be a population of cells amenable to neuroprotective strategies.

Although these data suggest there is little difference in infarct formation between 4-, 7, and 15-minute CCAo times, they do not suggest that there is no difference at all. We only examined the infarct area with TTC in the representative images in Figure 5.4, and TUNEL staining was not examined in other regions. It has been suggested that longer CCAo times replicate a model of global ischemia, a rare subset of ischemic stroke that affects the whole brain. Global ischemia models cause cell death in the hippocampus, so although not examined in this study, longer CCAo time (such as 15 minutes) may replicate conditions of global ischemia and cause unwanted memory deficits as a result of hippocampal cell death.

Chapter 6 - Mouse iPS Cells: Culture and Neuronal Differentiation

Introduction

iPS cells representative a remarkable step forward for stem cell research. iPS cells are a pluripotent cell source that overcomes the moral and ethical hurdles posited by embryonic stem cells (which are taken from a developing embryo) that have immense potential for research and for medicine. In the lab, they are tools for regenerative medicine and can serve as models of disease *in vitro.* In the clinic, they can possibly serve as a component of personalized medicine. Fibroblasts from a patient could be reprogrammed (Takahashi, Tanabe et al. 2007, Dimos, Rodolfa et al. 2008), differentiated, and transplanted into the same patient as cells, tissue, or perhaps even an organ.

The use of iPS cells for the present study necessitates successful differentiation of pluripotent iPS cells into neural progenitors that can terminally differentiate into cells of neural lineage: neurons and glia. We demonstrate in the following chapter mouse iPS cells can be efficiently differentiated into neurons that express NeuN and fire action potentials. These cells also can be tracked *in vivo* and we observe successful differentiation into NeuN-expressing neurons *in vivo* (results in the next chapter).

Current studies of iPS transplantation focus on differentiation into neurons and functional recovery after stroke (Oki, Tatarishvili et al. 2012, Mohamad, Drury-Stewart et al. 2013, Yuan, Liao et al. 2013, Chau, Deveau et al. 2014). Few studies examine terminal neuronal phenotype, or other cellular characteristics such as

growth factor and chemokine expression. We demonstrate in this chapter that iPS cells not only differentiate into neurons that fire action potentials and express neuronal markers, but these cells express growth factors and other pro-migratory proteins as well.

Last, the goal of this study is to examine the effect of FAK upregulation on aspects of cellular development that may benefit iPS transplantation into the ischemic brain. There have been no reports on endogenous FAK levels in iPS-derived neural progenitors. Before upregulation studies are to be undertaken, it is first necessary to characterize endogenous FAK expression in these cells, which is reported in the following chapter.

Results

Cell Culture Overview

Figure 6.1 illustrates a summary of the cell types used in the present study. We examined growth rate, cell density, and transfection potential. All of these parameters were necessary to gain an understanding of the individual cell types for successful culture moving forward.

Cell Type	Time to Confluency	Cells/Dish	Transfectable
HEK	4-5 days (1:10)	8.0-10.0*10^6/10 cm	Yes - High
CHO	2-3 days (1:10)	2.0-4.0*10^6/10cm	Yes - ?
MSC	3-5 days (1:3)	1.0-3.0*10^6/10cm	No - <5%
FAK Fibroblast	2 days (1:10)	4.0-8.0*10^6/10cm	Yes - High
MEF	2-3 days (1:5)	~10*10^6/15cm	No - <5%
1° Cortical Neurons	n/a	1*10^6/3.5mm	Yes - low
mIPS	2 days (1:8)	4.0-10.0*10^6/t25	Yes - medium

Figure 6.1. Cell Culture Summary.

Summary of cells used in the present study including their growth rate, cell density, and transfection efficiency.

Mouse iPS Cell Culture

Figure 6.2 presents an overview of mouse iPS cell culture. For the experiments described in this dissertation, pluripotent colonies were expanded on gelatin (**Figure 6.2.A**) and transferred to a rotary suspension culture for differentiation. Following 8 days of a 4-/4+ retinoic acid differentiation protocol, neurospheres (**Figure 6.2.B**) were harvested, dissociated into single cells, and plated on PDL-laminin coated dishes in SATO medium for terminal differentiation. Tuj-1 expression is prominent 3 days after plating with some Neurofilament expression as well (**Figure 6.2.C**). Immunostaining data on day 6 after plating demonstrates cells express NeuN and Neurofilament (**Figure 6.2.D**). NeuN expressing cells fire action potentials and show inward/outward currents characteristic of neurons that are sensitive to TTX. Results description and proceeding **Figure 6.2** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

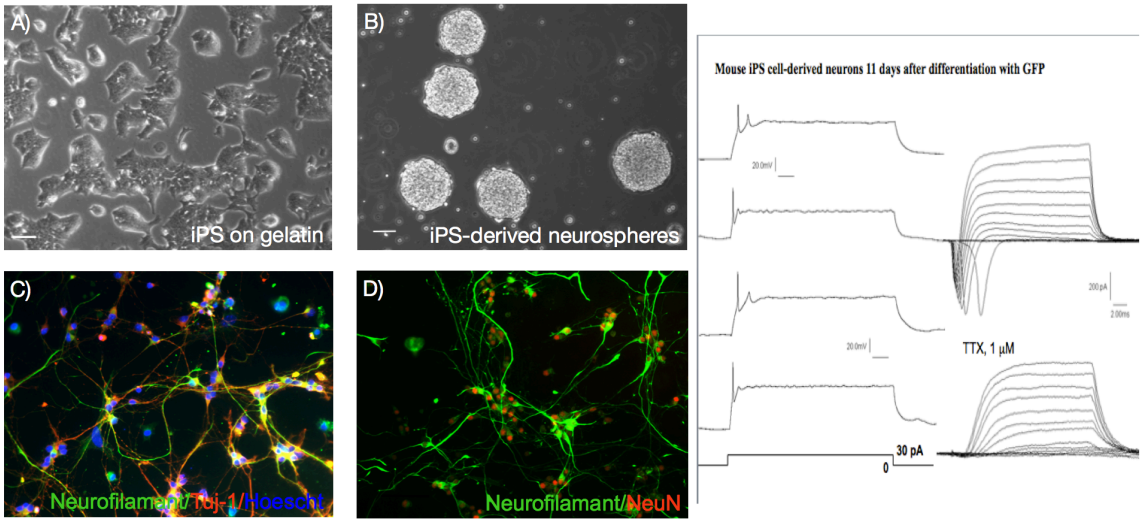


Figure 6.2. Mouse iPS Cells Differentiate Into Functional Neurons.

Mouse iPS cells are expanded on gelatin (A) and differentiated in rotary culture (B). 3 days after neurosphere plating and harvest, cells exhibit neuron-like morphology and express neuronal markers Tuj-1 and NF (C). 6 days after harvest and plating, cells express NeuN (D). Cells fire action potentials and exhibit TTX-sensitive Na+ currents (above).

Neuronal Differentiation Efficiency

Immunostaining data reveal that 92% of cells are Tuj-1 positive 3 days after plating. Tuj-1 is an immature neuronal cytoskeletal marker (**Figure 6.3.B**). Immunostaining data 6 days after plating reveals significant NeuN and neurofilament expression, and that at least 90% of all cells (calculated against total Hoechst positive cells) are NeuN positive (**Figure 6.4**). Results description and proceeding **Figures 6.3** and **6.4** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

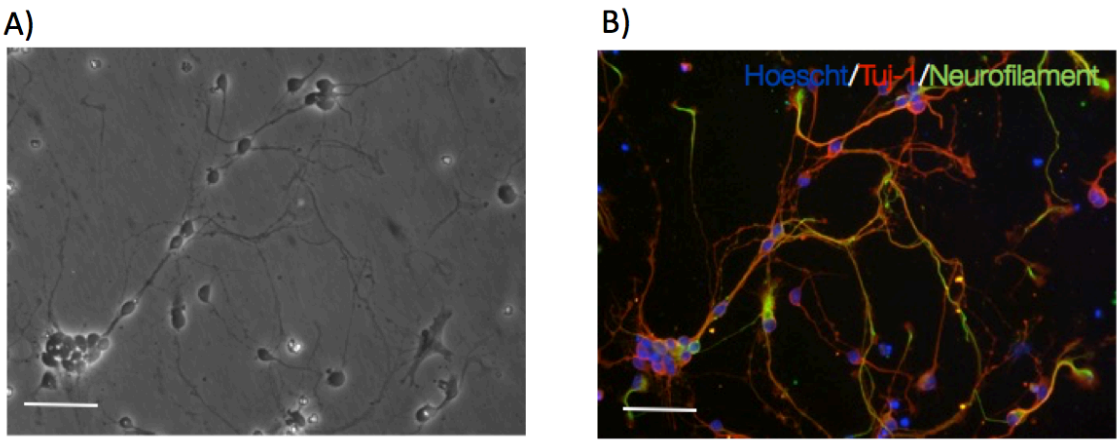


Figure 6.3. iPS-Derived Neurons Express TUJ-1 and NF.

Day 3 mouse iPS neurons (phase contrast, A) are stained for immature neuronal cytoskeletal marker Tuj-1 (aka β-III-tubulin), mature neuronal cytoskeletal marker Neurofilament (NF), and Hoechst (B). Counting Tuj-1 positive cells as a percentage of Hoechst positive cells reveals 92% of cells are Tuj-1 positive.

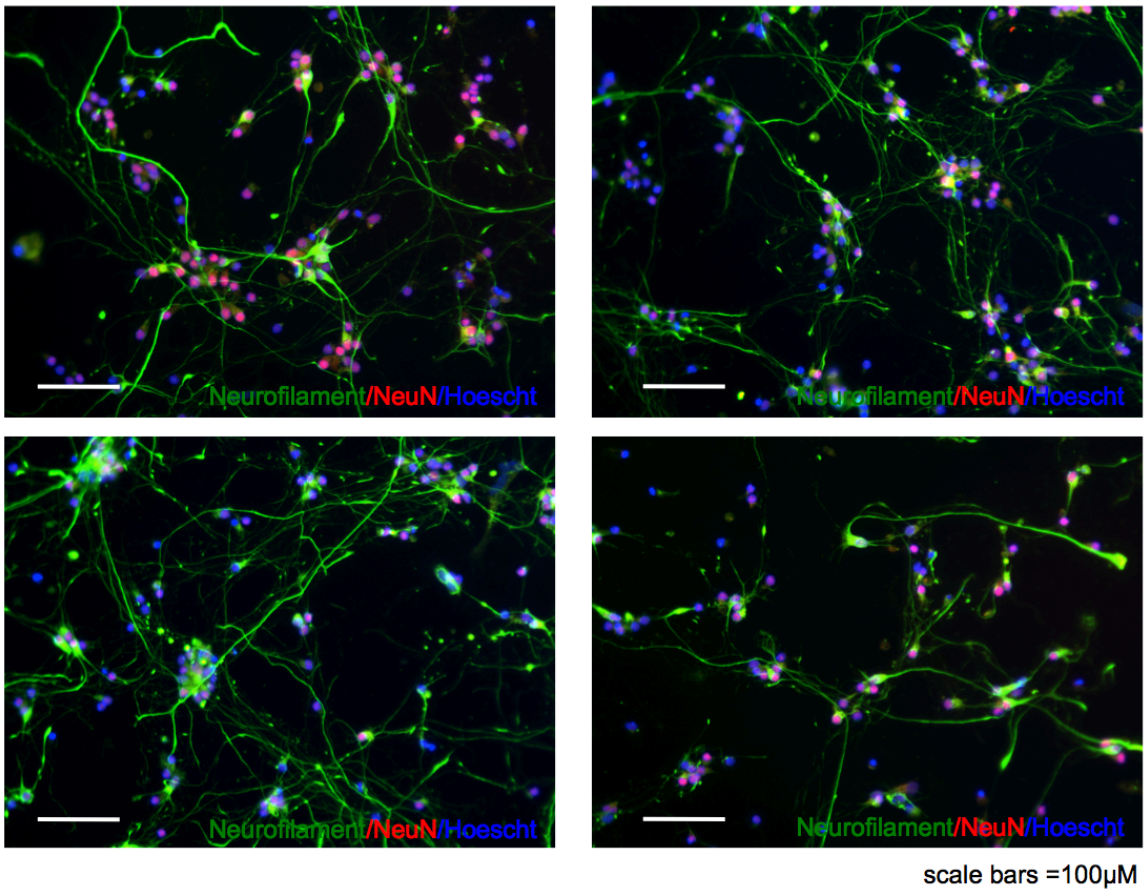


Figure 6.4. iPS-Derived Neurons Express NeuN.

Day 6 mouse iPS neurons are stained for mature neuronal cytoskeletal marker neurofilament (NF), nuclear marker NeuN, and Hoechst. Counting NeuN positive cells as a percentage of Hoechst positive cells reveals 90% of cells are NeuN positive. Four representative images are shown.

Mature Neuronal Markers

In addition to NeuN/neurofilament expression and action potential firing, cells express additional mature neuronal markers. SNAP-25, a marker of synaptogenesis, is expressed by NeuN-expressing iPS-neurons (**Figure 6.5.A**). These IHC data suggest synapse formation is potentially occuring between iPS-neurons *in vitro*. Additionally, iPS-neurons express the outward rectifying potassium channel Kv2.1 (**Figure 6.5.B**). This channel is paramount in repolarization of the neuron following voltage gated sodium channel activation. Results description and proceeding **Figure 6.5** is present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

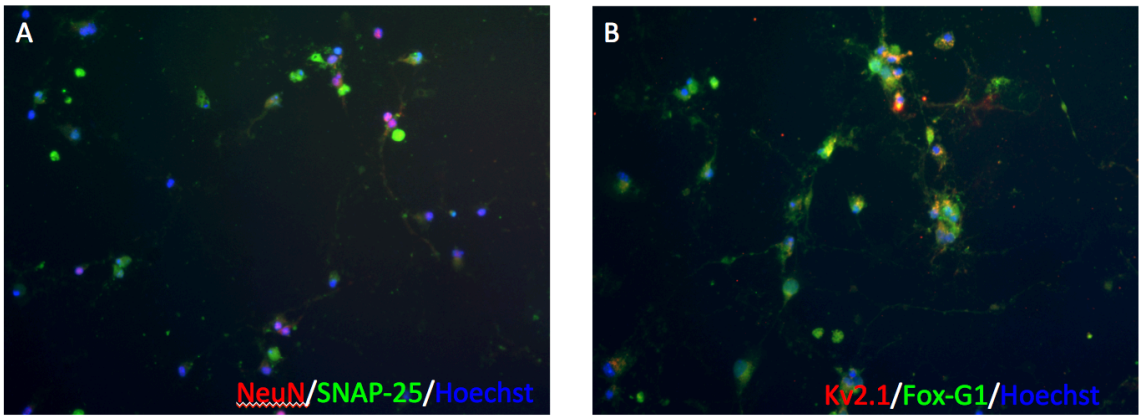


Figure 6.5. iPS-Neurons Express Additional Mature Neuronal Markers.

Day 10 iPS-derived neurons demonstrated expression of synaptogenic protein SNAP-25 (A), potassium channel Kv2.1 (B), and cortical forebrain marker Fox-G1 (B).

iPS Neuron Phenotyping

IHC demonstrates that the overwhelming majority of NeuN-expressing iPS neurons also express Fox-G1 (**Figure 6.5.B**). Fox-G1 is a transcription factor expressed by the embryonic telencephalon. Defects in Fox-G1 (such as Fox-G1 syndrome) lead to severe developmental defects and an underdeveloped cerebrum with low white matter density. These data suggest that the 4-/4+ protocol employed in iPS cells is leading cells into a cortical developmental trajectory. We undertook additional phenotyping of iPS-NPCs and neurons using reverse transcriptase PCR. We looked at neurotransmitter synthesis genes (synthetases) as a marker for neuronal phenotype. PCR data shows no dopamine β-hydroxylase (DβH) or tyrosine hydroxylase (TH) expression (**Figure 6.6.B**). We also observed no choline acetyltransferase (Chat) expression or tryptophan 5-hydroxylase (T5H) expression (data not shown). Glutamic acid decarboxylase (GAD-67) expression increases through terminal differentiation, suggesting the possibility that the neurons generated are GABA-ergic (**Figure 6.6.A**). Expression data of excitatory neurotransmitter markers such as glutaminase and vGlut1 is currently lacking.

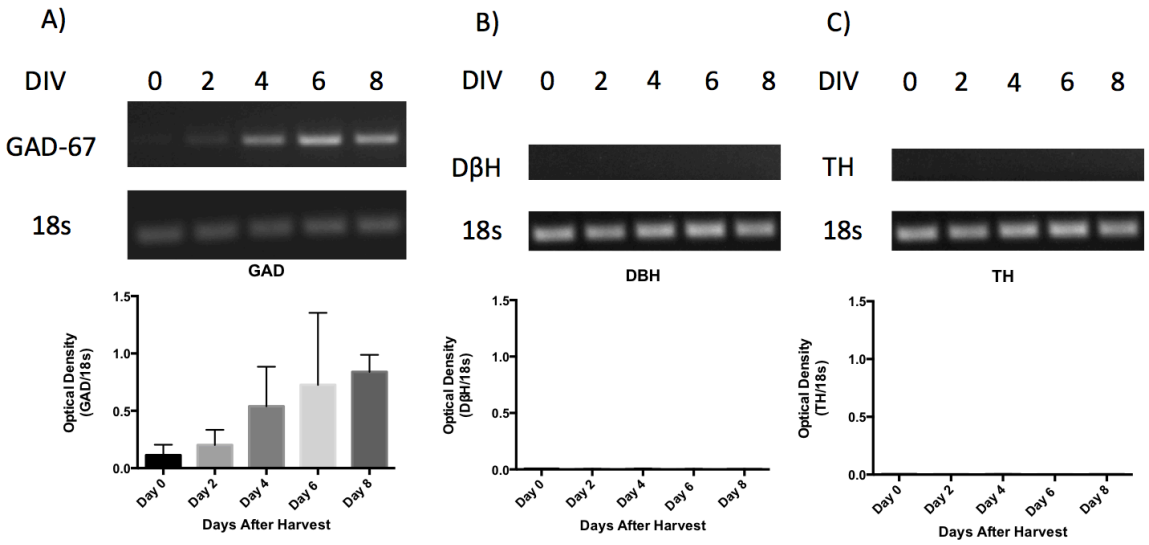


Figure 6.6. iPS-NPC Phenotyping.

Mouse iPS neural progenitors/neurons express GAD-67 through at least day 8 *in vitro* (A, n=3/group) but lack DβH (B) and TH (C) expression.

FAK Expression

We examined endogenous FAK expression to establish a baseline for measuring overexpression. PCR data shows abundant FAK expression at the messenger RNA level (Figure **6.7.A**). Levels increase slightly across terminal differentiation, but remain slightly lower than levels in the mouse cortex. At the protein level (**Figure 6.7.B**), FAK is expressed at high levels across terminal differentiation but peak at DIV 2. Phospho-FAK (FAK^{Y397P}) levels peak at DIV 2 and steadily decline thereafter. IHC demonstrates cellular localization of total FAK (**Figure 6.8**). FAK is located in the cell soma and cell processes. Phospho-FAK (FAK^{Y397P}) is also present at all time points examined in the cell cytoplasm and cell processes (**Figure 6.9**). Interestingly, it is absent from the cell nucleus. Using the polyclonal IHC antibodies on $FAK^{-/-}$ cells (which show no FAK signal via western blot, **Figure 6.10.A**), a signal is observed with FAK (**Figure 6.10.B**) and FAK^{Y397P} (**Figure 6.10.C**), which suggests that some of the signal observed may be non-specific. Staining patterns for FAK^{Y397P} do mirror patterns observed for GFP-FAK expression (shown in the proceeding chapter), which suggests that the staining patterns observed with these antibodies may not completely be non-specific binding. Results description and proceeding **Figures 6.7-6.10** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. Fak is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

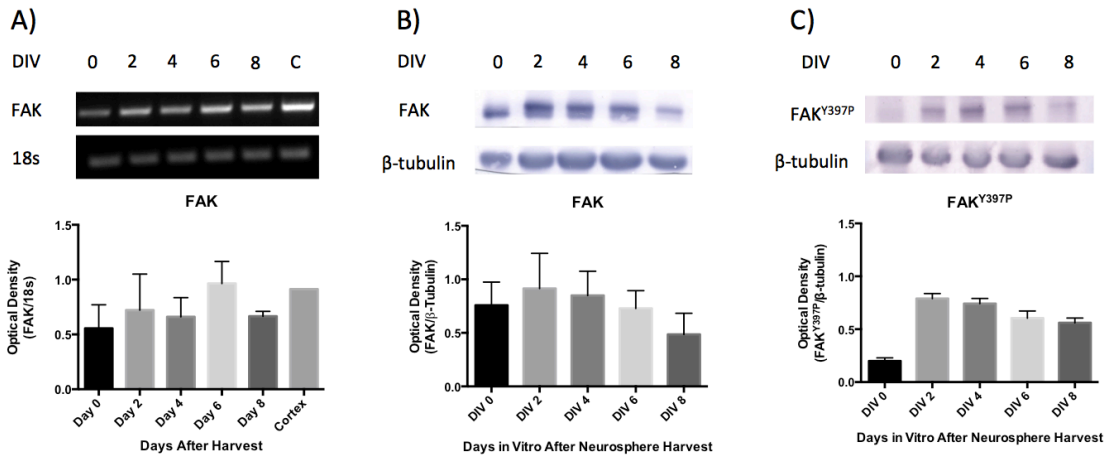


Figure 6.7. FAK Expression in iPS-Derived Neural Progenitors and Neurons.

Endogenous FAK expression was determined in iPS-derived neural progenitors and neurons aged from DIV 0 to DIV 8 using RT-PCR and immunoblotting. FAK is ubiquitously expressed at all time points at the mRNA (A) and protein level (B). Interestingly, activated FAK (FAK^{Y397P}, C) peaked at DIV 2 and steadily declined as cells aged.

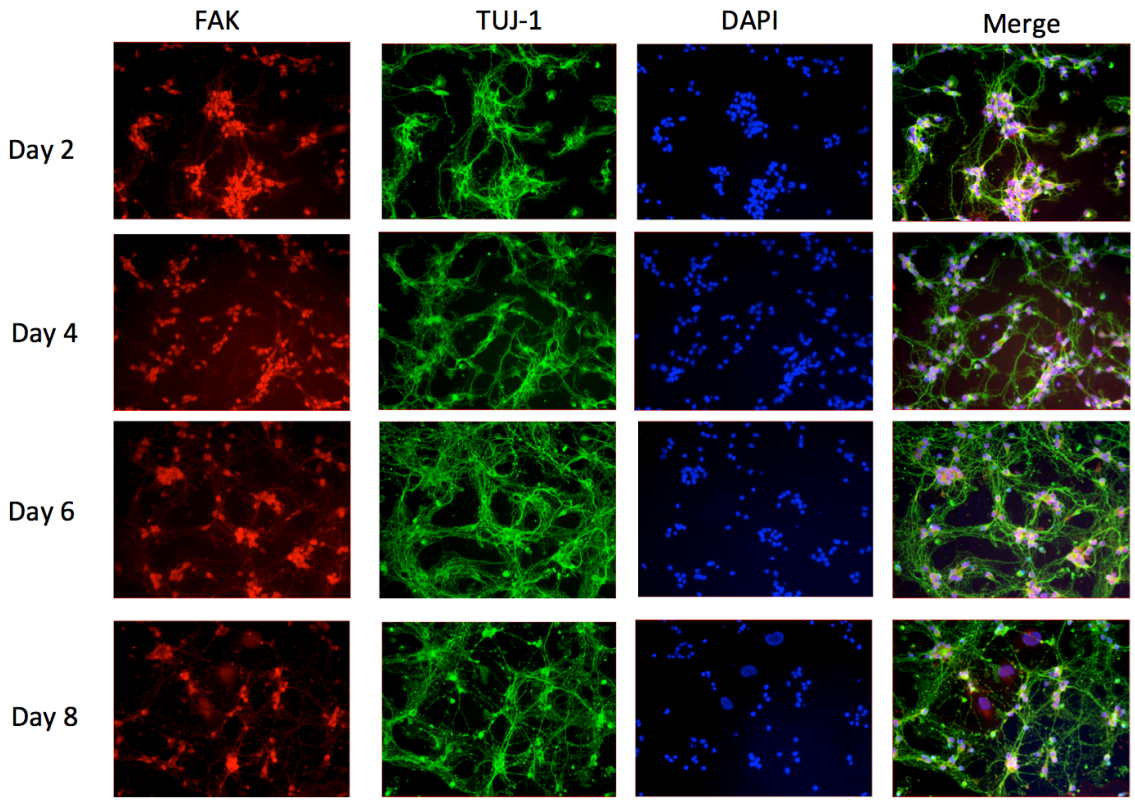


Figure 6.8. FAK Localization in iPS-Derived Neural Progenitors and Neurons.

Endogenous FAK expression was visualized with immunohistochemistry in iPS-derived neural progenitors and neurons. FAK was ubiquitously observed at all stages in the cell soma and cell processes.

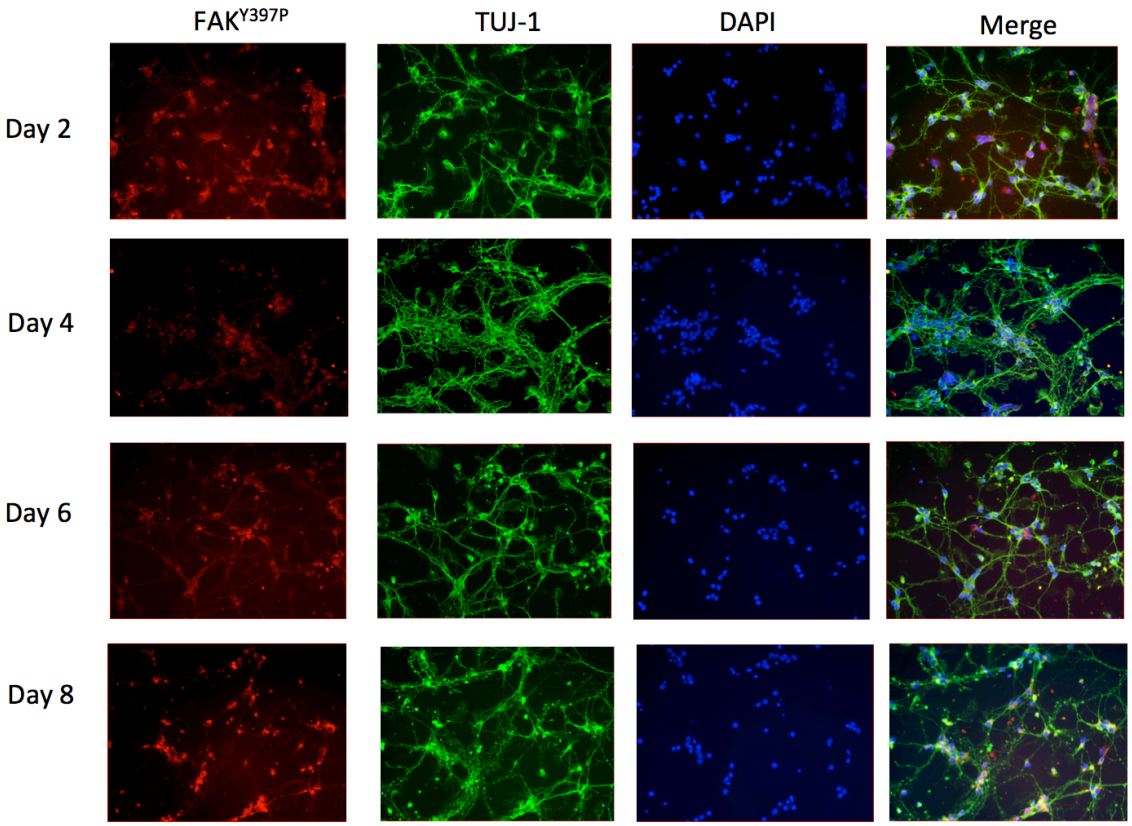


Figure 6.9. FAK^{Y397P} Localization in iPS-Derived Neural Progenitors and Neurons.

Endogenous FAK^{Y397P} (activated FAK) expression was visualized with immunohistochemistry in iPS-derived neural progenitors and neurons. FAK^{Y397P} was ubiquitously observed at all stages in the cell soma cytoplasm (absent in the nucleus) and cell processes.

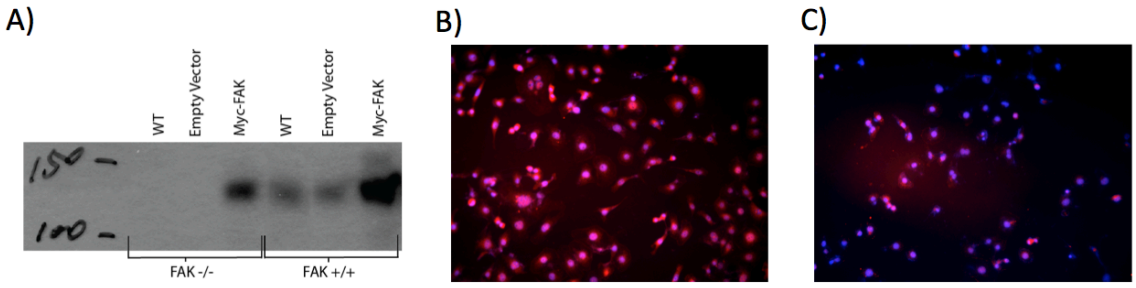


Figure 6.10. Non-Specific FAK, FAK^{Y397P} Staining in FAK$^{-/-}$ Fibroblasts.

FAK expression is absent in FAK-/- Fibroblasts (A), however IHC shows FAK (B) and FAK^{Y397P} (activated FAK, C) signals in these cells. These data suggest that some of the signals observed in the previous two figures may be non-specific, however staining patterns align with GFP-FAK expression (Figure 6.X), suggesting that not all of the signal is non-specific.

iPS-NPCs Express Pro-Migratory Proteins

Our goal is to test intranasal delivery of iPS-NPCs. In order to be effective, iPS-NPCs must migrate from the nasal cavity to the ischemic infarct. It is not clear what mechanisms cells use to gain entry into the CNS and how cells home to the ischemic infarct, however the CXCR4/SDF-1α axis is suggested to be involved. IHC data show that iPS-NPCs express CXCR4 (**Figure 6.11.A**) and doublecortin (DCX, **Figure 6.11.B**), which are crucial for neural progenitor migration in response to SDF-1α. Immunoblotting data demonstrates that CXCR4 is expressed at high levels across terminal differentiate and that its expression doesn't diminish over time (**Figure 6.12.A, 6.12.B**). Additionally, iPS-NPCs express MMP-9 in its pro- and active forms (**Figure 6.12.A, 6.12.C-E**). MMP-9 is crucial for aiding migration through local extracellular matrix (ECM) remodeling. Results description and preceeding **Figures 6.11** and **Figure 6.12** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

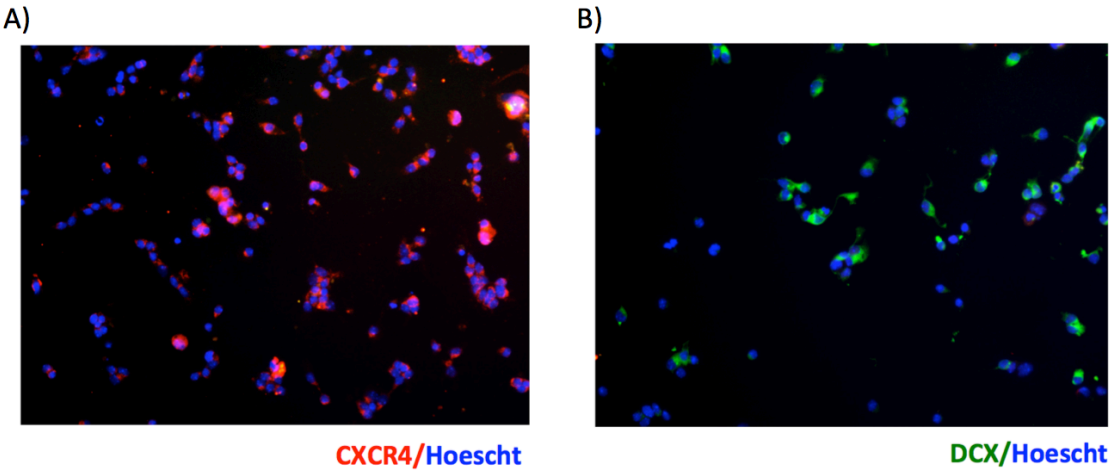


Figure 6.11. iPS-NPCs Express Pro-Migratory Proteins CXCR4 and DCX.

iPS-NPCs express CXCR4 (A), the complement receptor to SDF-1α, and doublecortin (DCX, B), a protein associated with migrating endogenous neural progenitors, 1 day after neurosphere dissociation and plating. These data support the notion that iPS-NPCs may migrate towards SDF-1α, which is upregulated and secreted by injured cortical tissue.

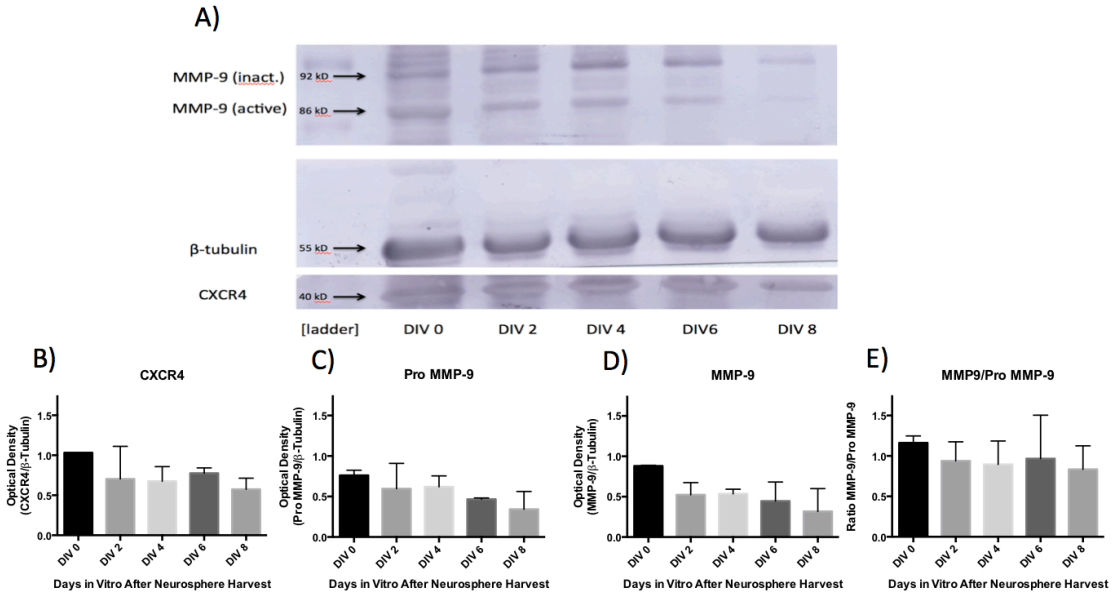


Figure 6.12. iPS-NPCs and Neurons Express CXCR4 and Active MMP-9.

Immunoblots show migratory factor levels in iPS-NPCs and iPS-neurons (A). iPS-NPCs express CXCR4, the complement receptor to SDF-1α, at high levels at all time points after plating and terminal differentiation (B). Cells express MMP-9, visualized as Pro-MMP9 (C), active MMP-9 (D), and as a ratio of active/total (D).

Growth Factor and Cytokine Expression

We have previously demonstrated growth factor expression by mouse iPS-NPCs but these data lack a temporal component. PCR data demonstrate that iPS-NPCs express brain-derived neurotrophic factor (BDNF, **Figure 6.13.A and 6.13.B**) that peaks at DIV 8. Vascular endothelial growth factor (VEGF, **Figure 6.13.A and 6.13.C**) expression is also present in iPS-NPCs across terminal differentiation. We also examined stromal cell-derived factor 1-α (SDF-1α) expression, however no expression was present (**Figure 6.14.A and 6.14.B**). Results description and preceeding **Figures 6.13** and **Figure 6.14** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

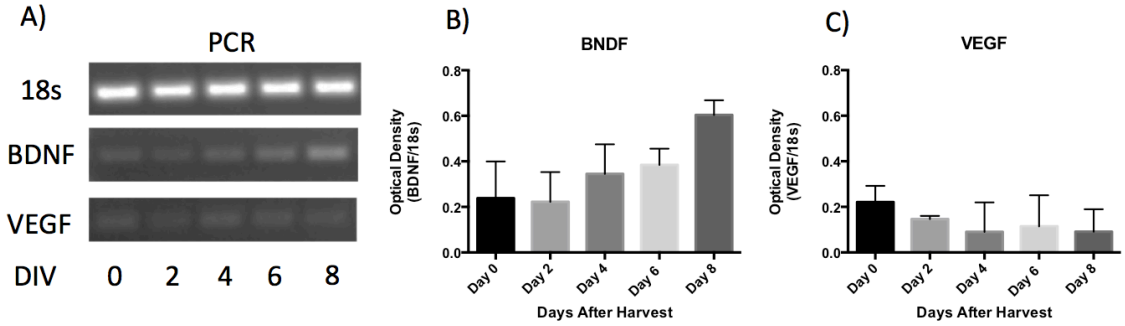


Figure 6.13. iPS-NPCs and Neurons Express BDNF and VEGF.

Mouse iPS neural progenitors/neurons express growth factors BDNF (A, B) and VEGF (A, C) through at least day 8 *in vitro* (n=3/group). Growth factor production may improve endogenous neurovascular repair.

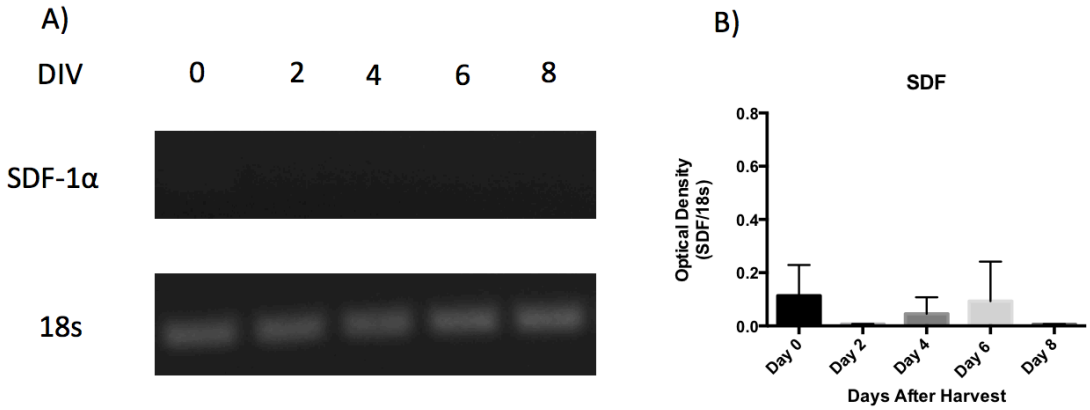


Figure 6.14. SDF-1alpha Expression in iPS-NPCs and Neurons.

iPS-NPC transplantation increases the number of BrdU positive cells in the ischemic penumbra. One possible mechanism behind this observation is that the transplanted iPS-NPCs express and secret additional SDF-1α that could attract endogenous neural progenitor cells to the injured brain. RT-PCR data above (A, B) show that cells express minimal SDF-1α through 8 days in culture, which suggests an alternative mechanism for increased Brdu positive cells in the penumbra after transplantation.

Discussion

Here we have demonstrated that mouse iPS cells can be efficiently differentiated into neurons that express mature neuronal markers and fire action potentials. Cells express synaptogenic markers, ion channels, and cortical markers *in vitro*. PCR data demonstrates lack of Chat, DβH, TH, and T5H expression. This suggests that iPS-derived neurons are not cholinergic, noradrenergic, dopaminergic, or serotonergic. These data are consistent with Fox-G1 expression data because these types of neurons are most present in deeper brain structures that are not of telencephalic origin. PCR data suggests that cells are GABA-ergic, although excitatory marker data is currently absent and needs further investigation.

PCR, immunoblot, and IHC data demonstrate that FAK is expressed at high levels at all stages of terminal differentiation in iPS-NPCs. Immunoblots show that FAK and FAK^{Y397P} expression peaks at DIV 2, which coincides with observations of prominent neurite outgrowth at these times points. These data suggest that the role of FAK in iPS-NPCs may be most important in neurite outgrowth. Staining data demonstrate that FAK is localized ubiquitously throughout the cell (in the soma and processes), however FAK^{Y397P} signal is absent in the nucleus. Several studies have demonstrated a role of FAK in nuclear signaling. However, the present staining data suggest that FAK^{Y397P} in iPS-NPCs and neurons is not contributing to nuclear signaling. Significant difficulty in obtaining successful FAK IHC was observed in this study, using several antibodies and protocols previously described by many studies. Successful FAK staining was finally obtained with polyclonal antibodies from Santa

Cruz. Staining using these antibodies in FAK-/- cells suggest that some of the signal observed in the iPS-NPC staining may be non-specific. The signal observed in iPS-NPCs (especially for FAK^{Y397P}) does mirror GFP-FAK signal observed in the proceeding chapter though, suggesting that the FAK signal in Figures 6.8 and 6.9 may not be so non-specific.

We have confirmed with IHC and immublotting that iPS-NPCs express pro-migratory proteins, which may be essential for success of intranasal administration. CXCR4 and DCX expression is observed via IHC, similarly to migrating endogenous neuroblasts. Immunoblots demonstrate that CXCR4 expression is sustained at high levels through terminal differentiation. These data demonstrate that these cells should be able to detect and home to SDF-1α expressing cells in the ischemic infarct at least 8 days after transplantation. Cells also express MMP-9 in the pro- and active forms across terminal differentiation. MMP-9 expression may aid cellular migration of iPS-NPCs through endogenous ECM remodeling around the cellular microenvironment of transplanted cells.

Finally, we have demonstrated previously that transplanted iPS-NPCs can increase endogenous neurogenesis and angiogenesis (Chau, Deveau et al. 2014). We observe an increase in BrdU positive cells in the ischemic penumbra with transplantation. In the present study, we examined SDF-1α expression to further elucidate the mechanism of increased BrdU following transplantation. iPS-NPCs lack SDF-1α at the mRNA level at all time points of terminal differentiation. These data suggest that they may not produce or secrete additional SDF-1α *in vivo* that can

attract endogenous migrating neuroblasts and endothelial progenitor cells to the ischemic infarct. Additional study is needed to elucidate this mechanism.

Chapter 7 - Efficient and Stable Transgenic iPS Cell Creation

Introduction

iPS cells are perhaps the most promising stem cell type to date for the advancement of stroke therapies. They can efficiently be differentiated into neurons for transplantation, increase endogenous angiogenesis and neurogenesis (Chau, Deveau et al. 2014), express growth and pro-migratory factors (see previous chapter), and have the potential for personalized medicine. While we are enthusiastic about studies to date that show the potential of iPS therapy for stroke, many questions remain and their use is currently not optimal.

Previous studies have focused on whether or not iPS therapy can improve behavioral outcomes in rodent models of ischemia (Oki, Tatarishvili et al. 2012, Mohamad, Drury-Stewart et al. 2013, Yuan, Liao et al. 2013, Chau, Deveau et al. 2014). Several studies demonstrate that cells terminally differentiate into neurons that could replace dead cells in the ischemic brain (Mohamad, Drury-Stewart et al. 2013, Chau, Deveau et al. 2014), and at least one study demonstrates that these cells can integrate into existing host circuitry after stroke (Yuan, Liao et al. 2013). Additional studies are needed that focus on the fate of transplanted cells. Questions currently remain such as the phenotype of transplanted neurons, how many survive, how many integrate, the long term effects, etc. Efficient cell tracking methods are essential to to answer these questions. Previous cell tracking methods rely on pre-incubation of transplanted cells with Hoechst (Wei, Cui et al. 2005, Mohamad, Drury-Stewart et al. 2013). This method is fast, cheap, and efficient. However this

method is unreliable as the Hoechst dye leaks from dead transplanted cells and is taken up into endogenous cells. Other labeling paradigms such as membrane-bound quantum dot labeling have been attempted by our lab without success. Another method to trace cells would be to induce expression of a fluorescent marker protein, such as GFP, by the cells. This is common in research, however description and production of stable GFP-producing iPS cells and iPS-derived neurons is lacking in the literature. We demonstrate in this chapter the production of stable GFP and mCherry producing mouse iPS cells that express the exogenous marker at all stages of differentiation without interfering with pluripotency or differentiation.

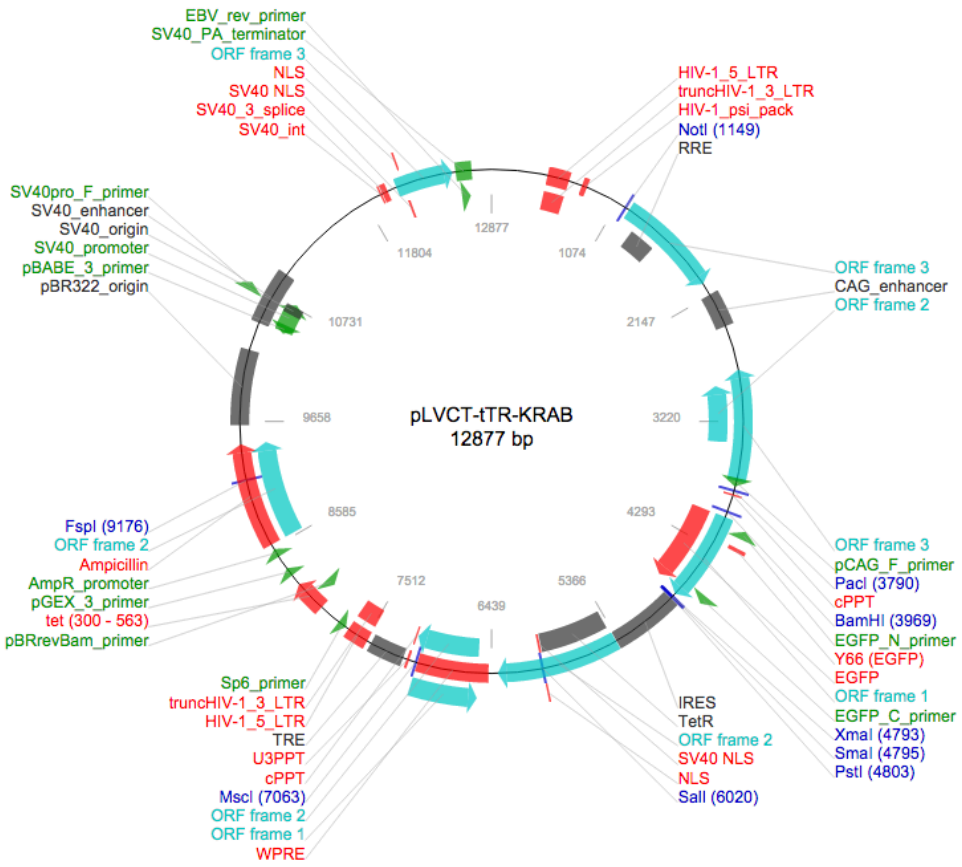
We have discussed ways in which current transplantation methods are not optimal. Low cell survival and low migration into the injured region of the brain are two examples that demonstrate hurdles to clinical translation. Genetic overexpression and manipulation of cells before transplantation is one possible way to overcome these issues. This strategy is currently not reported in the literature for iPS cells. Focal adhesion kinase (FAK) is an excellent target protein to overexpress because of its involvement in cell survival, adhesion, migration, and neurite outgrowth. Because of its relation to tumor metastasis and the potential of iPS tumorigenesis after transplantation, constitutive FAK upregulation may not be ideal. We demonstrate for the first time in the following chapter inducible and constitutive methods for overexpressing FAK in mouse iPS cells, and that the development of FAK overexpressing mouse iPS cells is possible.

Results

Inducible Expression Construct Design

One of the concerns facing iPS transplantation is the possibility of tumorigenesis of undifferentiated transplanted cells. Although we have not yet observed this *in vivo* (as long as 6 months after transplantation), it is a real concern. FAK is related to increased migration and motility of tumor cells leading and contributing to metastasis. In the long term, constitutive FAK expression may not be ideal and adding an on/off "switch" to control FAK overexpression may be advantageous. We designed inducible FAK expression constructs where FAK expression can be induced with doxycycline. The pLVCT expression backbone was chosen because transgene expression is placed under a promoter that is transcriptionally active in mammalian cells and can be highly expressed and tightly controlled with doxycycline addition. Construct designs are shown in **Figure 7.1**.

A)



B)

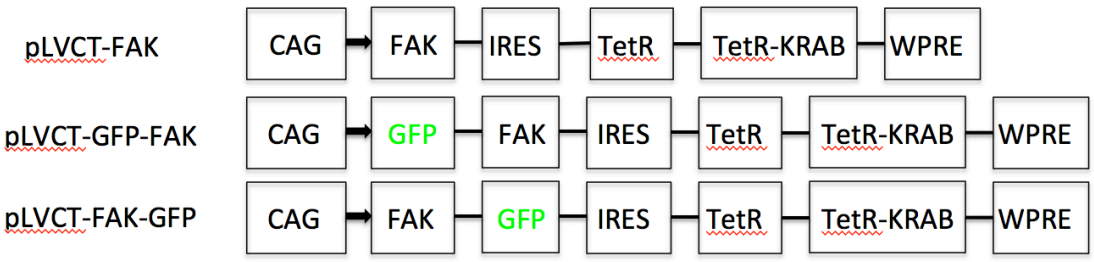


Figure 7.1. Inducible FAK Expression Vector Design.

The pLVCT backbone (pLVCT-GFP, A) was used as a basis for inducible vector design and construction. Inducible construct designs are shown in (B).

Inducible Construct Validation

Clones were created by the Emory University Cloning Core. Clones were amplified and expression was validated in mouse fibroblasts (**Figure 7.2**) before moving forward with iPS cells. Clones were transiently transfected into cells and doxycycline was added for 48 hours. GFP-FAK expression can be observed and increases with doxycycline addition in concentrations ranging from 50ng-12500ng/μL.

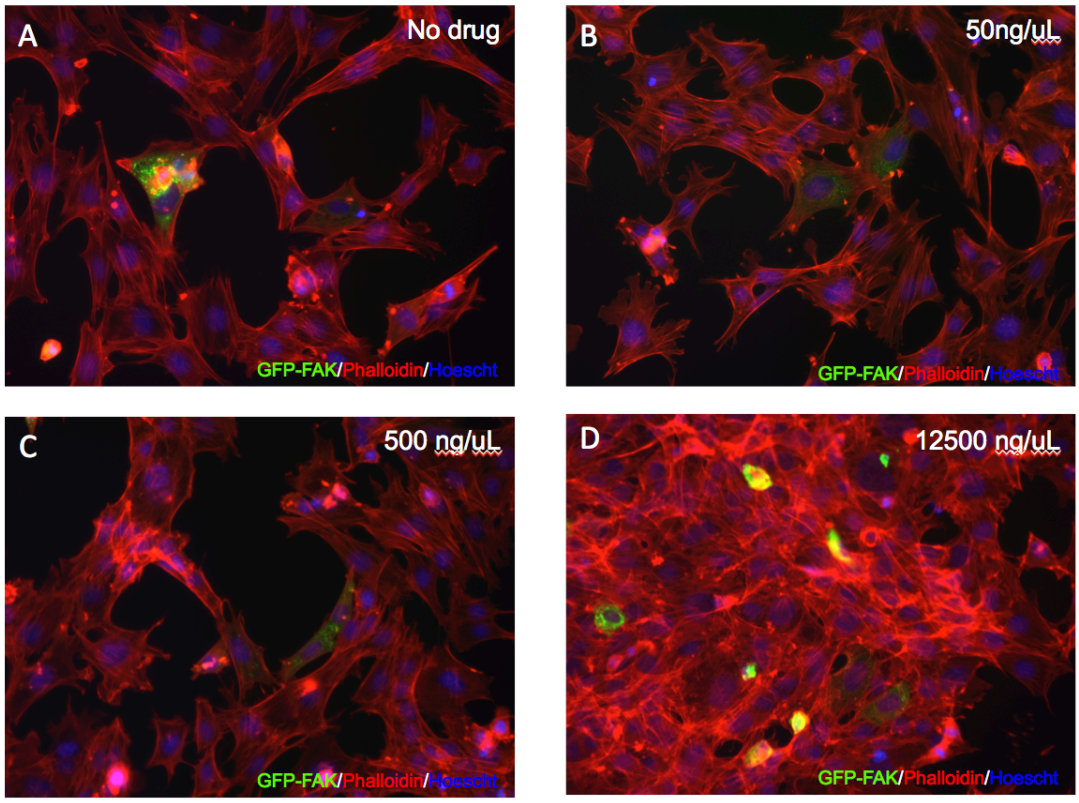


Figure 7.2. Inducible FAK Expression Vector Validation in Fibroblasts.

Mouse fibroblasts show GFP-FAK expression after transfection with an inducible FAK vector. Expression appears to increase after increasing dosages of doxycycline (A-D), which is to be expected.

iPS Transfection Optimization

After constructs were verified, they were then tested and validated in mouse iPS cells. Transfection of mouse iPS cells is currently not reported and protocols did not exist. In order to validate constructs in iPSCs and undergo stable colony selection, transfection was first optimized. Optimization of pluripotent iPSC transfection was undertaken (**Figure 7.3**) with varying ratios of DNA:lipofectamine and varying seeding densities of iPSCs.

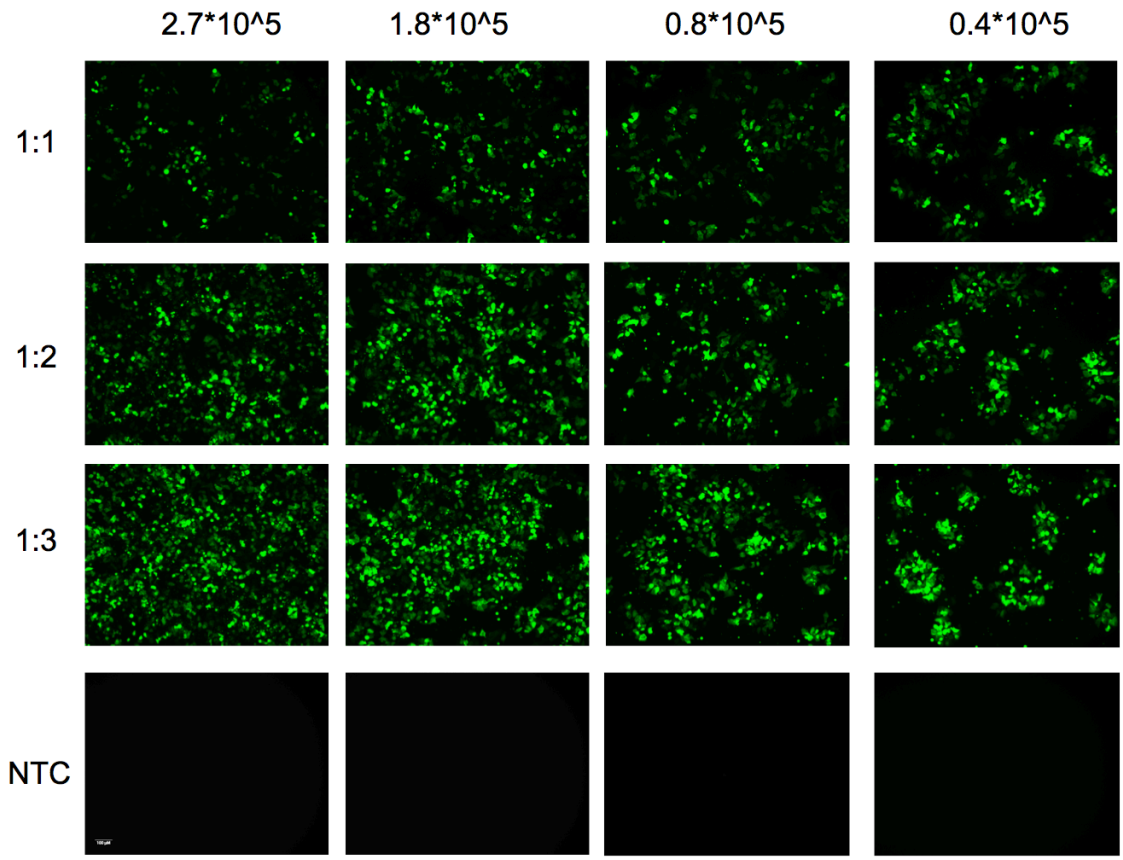


Figure 7.3. Mouse iPS Transfection Optimization.

Transfection is a commonly used technique to express transgenes in mammalian cells, however is currently not reported in pluripotent mouse iPS cells. Optimization of the procedure in terms of seeding density (cell number) and DNA:lipofectamine ratio was necessary before moving forward with further experiments in this cell type.

Transfection of miPSCs and Doxycycline Induction

Pluripotent mouse iPSCs were transfected with a constitutive pCMV-myc-FAK control construct and the pLVCT-FAK and pLVCT-GFP-FAK constructs (**Figure 7.4**). Western blotting shows increased FAK levels in pLVCT-FAK and pLVCT-GFP-FAK transfected cells that almost reaches that of the constitutive control construct without doxycycline. These data show that the pLVCT-FAK and pLVCT-GFP-FAK constructs are leaky, undermining the utility of an inducible system. Additionally, because FAK is expressed at ubiquitously high levels, the overexpression level observed may not be enough to create the desired phenotype. **Figure 7.5** shows an increase in GFP-FAK expression with the addition of doxycycline in pLVCT-GFP-FAK cells, with an optimal doxycycline concentration of 50ng/μL. Doxycycline was added to cells for 48 hours. Although these data are promising, the size of the inducible construct (~15kb) results in inefficient transfection and the inability to create stable colonies based on transfection.

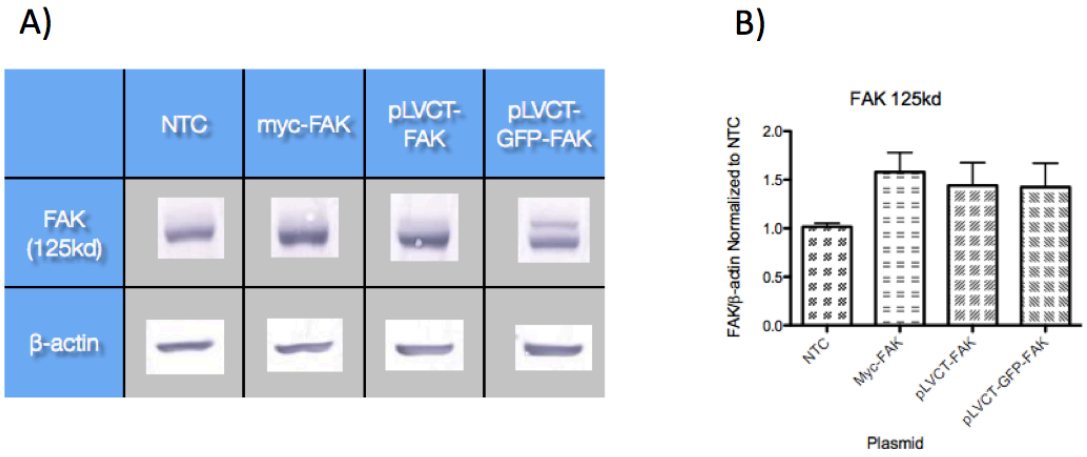


Figure 7.4. Transfection of Inducible Vectors into Pluripotent Mouse iPS-Colonies.

Transfection of inducible FAK vectors pLVCT-FAK and pLVCT-GFP-FAK appear to increase FAK levels as assayed with western blots (n=3; A, B). Expression was not induced with doxycycline, showing that even though FAK expression is increased, the vectors are "leaky".

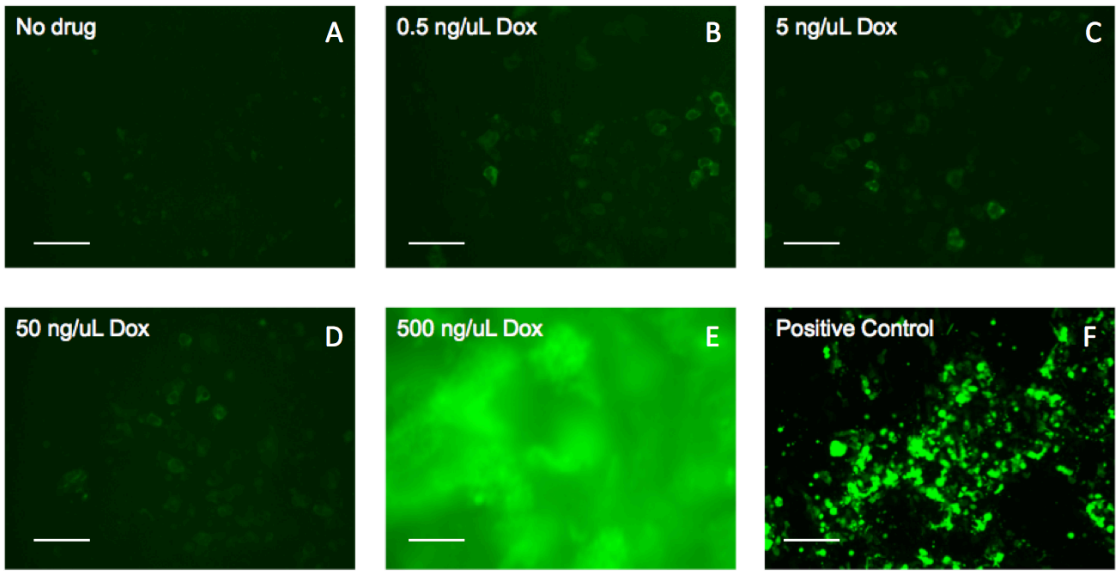


Figure 7.5. Doxycycline-Induced FAK Expression in Transfected Pluripotent Mouse iPSCs.

Pluripotent mouse iPS colonies were transfected with pLVCT-GFP-FAK and administered increasing dosages of doxycycline for 48 hours (0.5ng/μL-500ng/μL). GFP-FAK expression appears to increase with increasing concentrations of doxycycline. Concentrations of 500ng/μL (E) and up interfere with imaging and kill cells. (F) demonstrates pMAX-GFP transfection control.

Viral Induction of Mouse iPS Colonies

Because stable colonies of pLVCT-GFP, pLVCT-FAK, and pLVCT-GFP-FAK could not be created with transfection, the Emory University Viral Vector Core packaged these expression constructs into lentiviruses. Lentivirus was added to pluripotent miPS colonies for 48 hours and cells observed for 7 days thereafter (**Figure 7.6**). No GFP or GFP-FAK expression was observed. Because the constructs are inducible, we then added doxycycline (50ng/μL) for 5 days. Again, no GFP or GFP-FAK expression was observed (**Figure 7.7**).

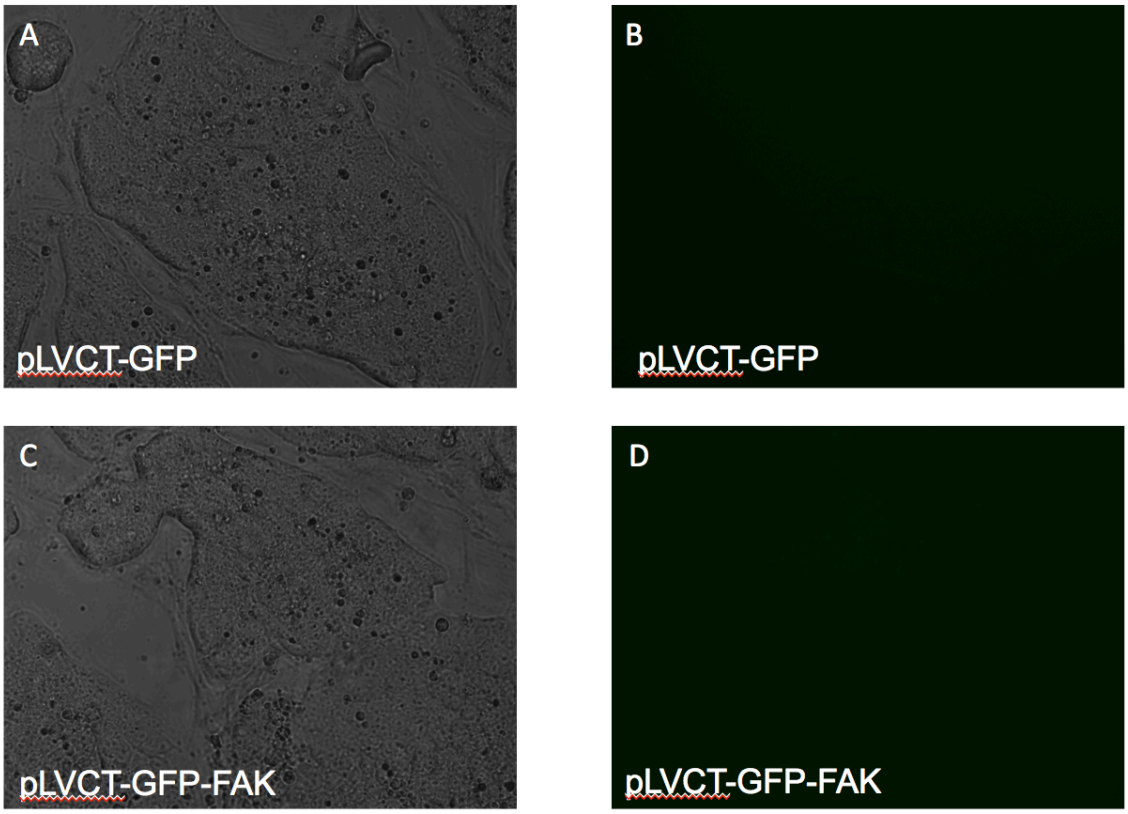


Figure 7.6. Addition of pLVCT-GFP and pLVCT-GFP-FAK Lentiviruses to Pluripotent Mouse iPS Colonies.

Lentiviruses packaged with the pLVCT-GFP and pLVCT-GFP-FAK expression vectors were adminstered to pluirpotent mouse iPS colonies (phase contrast A, C). No GFP expression was observed after 7 days.

Figure 7.7. Addition of Doxycycline to pLVCT-GFP and pLVCT-GFP-FAK Pluripotent Mouse iPS Colonies.

Pluirpotent mouse iPS colonies (representative phase contrast A) infected with lentiviruses packaged with the pLVCT-GFP and pLVCT-GFP-FAK expression vectors were adminstered doxycycline (50ng/μL) for 5 days. No GFP signal was observed for cells infected with the pLVCT-GFP (A, B) or pLVCT-GFP-FAK (C) lentiviruses.

Validation of Additional Expression Constructs

Because we were unable to create stable cells based on the pLVCT and pLVCT-derivatives, we validated additional constructs via transfection in mouse iPS colonies to be used as constitutively expressing backbones. Vector pEGIP showed the highest GFP expression (**Figure 7.8**). Because transgene expression for the present study relies on expression in the neuronal stage, we also validated this expression vector in primary cortical neurons. Transfection of pEGIP into primary cortical neurons demonstrated that this expression construct is transcriptionally active and produces GFP in these cells (**Figure 7.9**).

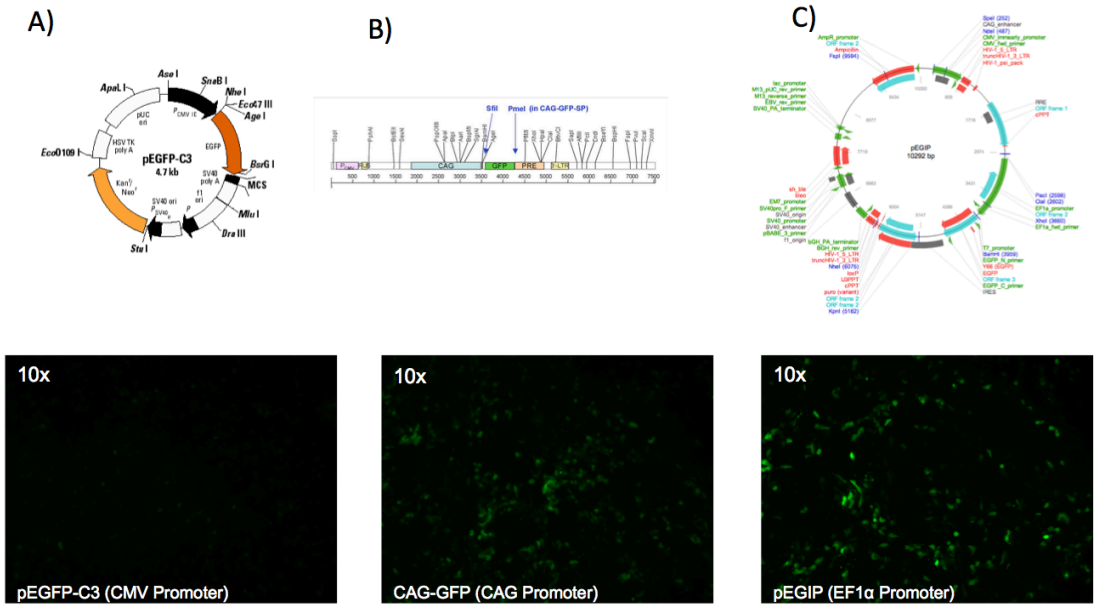


Figure 7.8. Validation of Constitutively-Expressing Lentiviral-Based Expression Vectors.

Due to the failure of the inducible viral-based approach to creating stable FAK-overexpressing cells, we focused attention on a constitutively-expressing lentiviral-based system with a mammalian drug-resistance cassette for easy stable colony selection. We tested 3 GFP-expressing vectors with transient transfection in order to determine the most efficacious expression vector in pluripotent mouse iPS-colonies: pEGFP-C3 (A), CAG-GFP (B, Addgene plasmid #16664), and pEGIP (C, Addgene plasmid #26777). The pEGIP vector demonstrated stronger GFP expression than the pEGFP-C3 and CAG-GFP expression vectors tested.

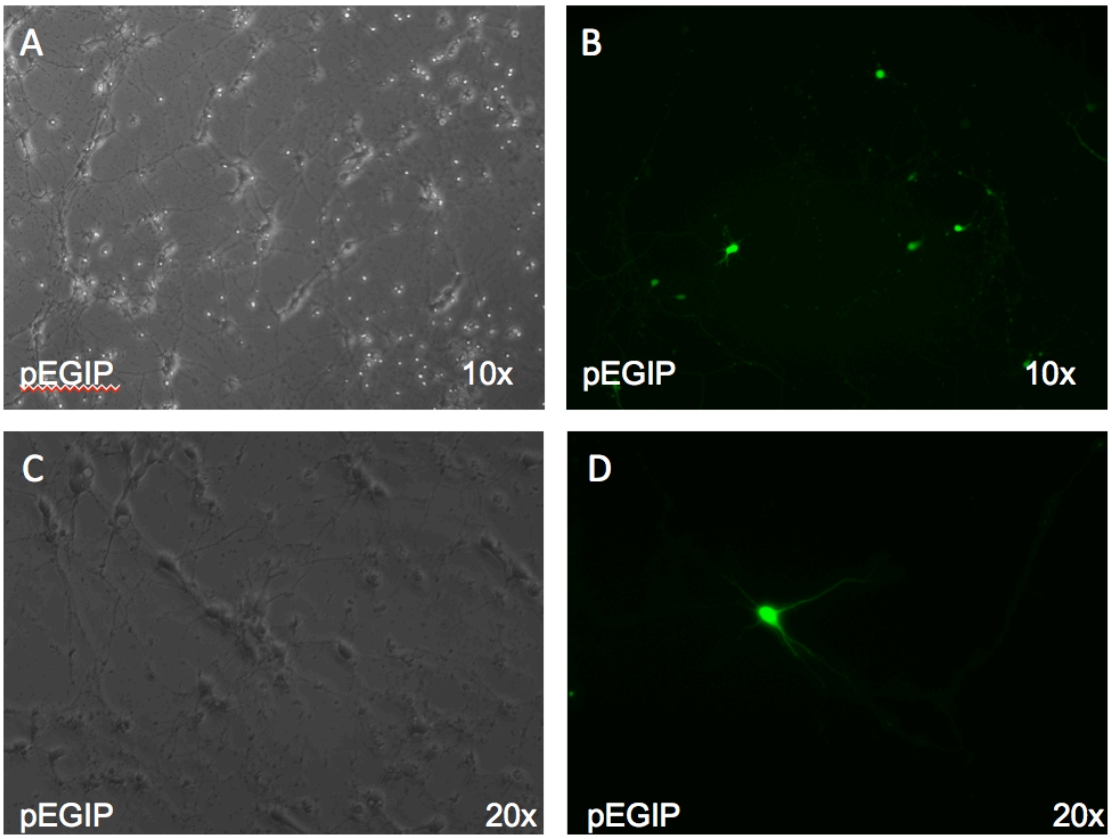


Figure 7.9. Validation of pEGIP in Cortical Neurons.

In order to promote the desired upregulation, stable transgenic iPS cells must express the transgene not only in the pluripotent stage, but in the terminally differentiated stage. We tested to see if the EF1α promoter in the pEGIP construct was transcriptionally active in primary mouse cortical neurons and if GFP would therefore be expressed. Mouse primary cortical neurons were transfected 3 days after plating and observed for GFP expression for an additional 5 days. Although transfection of primary cortical neurons is inefficient, we observe GFP expression in these cells as long as 5 days after transient transfection (A, C phase contrast, pEGIP/GFP B, D).

Lentivirus-Mediated Stable Cell Creation

Again, we were not able to create stable GFP expressing colonies with pEGIP vector and transfection. The Emory University Viral Vector Core packed the pEGIP vector into a lentivirus that was then used to infect pluripotent miPS colonies. Successful stable GFP expressing colonies were observed after viral infection and puromycin addition (**Figure 7.10**). IHC demonstrates that stable colonies still express pluripotency markers Sox-2, Pax-6, and Oct-3/4 (**Figure 7.11**). These data suggest that stable colony creation and maintenance does not interfere with pluripotency. GFP expression is maintained into the neurosphere stage (**Figure 7.12**), however by that point transgene expression is low. Following plating and terminal differentiation, transgene expression was not observable nor was observable following cell transplant *in vivo*.

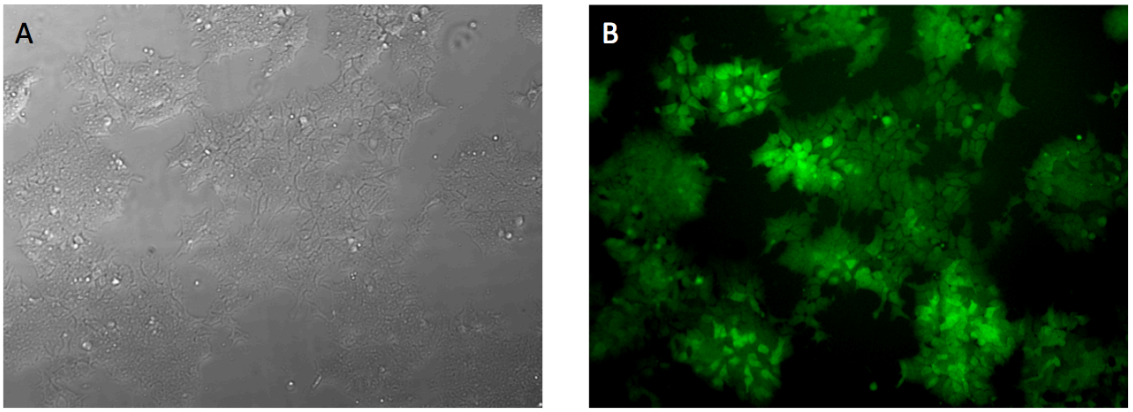


Figure 7.10. Stable Pluripotent pEGIP Mouse iPS Colonies.

pEGIP virus ($5x10^8$ IU/mL) was applied to pluripotent mouse iPS cells for 72 hours. Puromycin was used used to select stable GFP-expressing colonies. With puromycin selection, 100% of miPS cells expressed the GFP transgene.

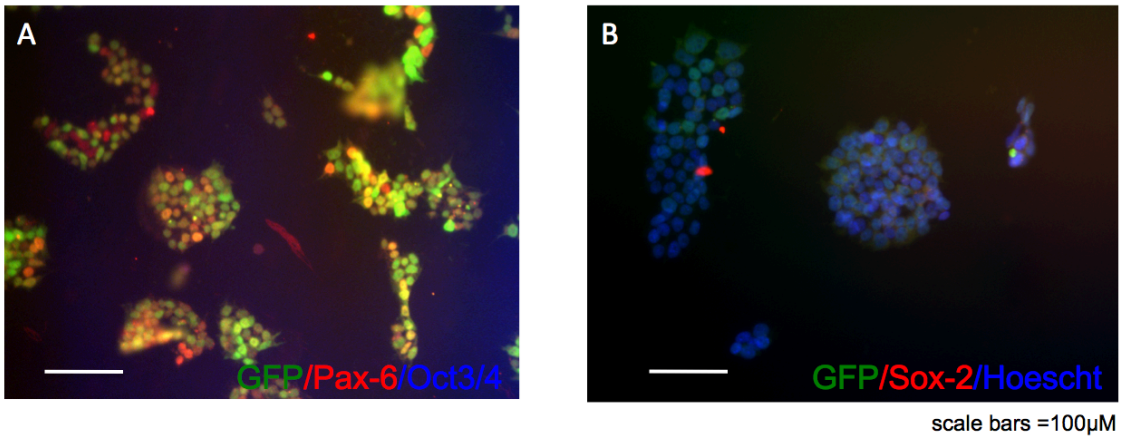


Figure 7.11. Stable pEGIP-iPS Colonies Retain Pluripotent Marker Expression.

A concern with additional genetic manipulation of existing iPS lines is interference with pluripotency. We stained stable pEGIP-iPS colonies for pluripotent markers Pax-6 (A), Sox-2 (B), and Oct3/4 (A); we observed expression of these markers is retained after infection with the pEGIP lentivirus, GFP expression, and puromycin selection.

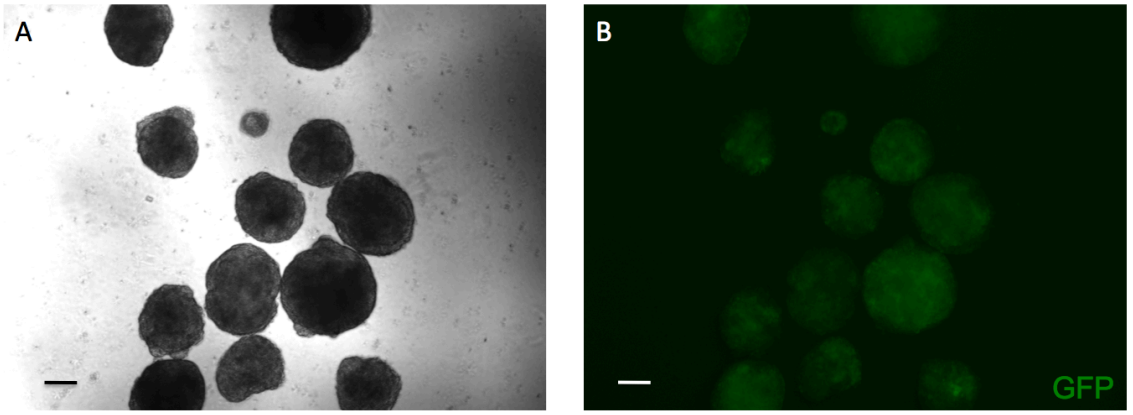


Figure 7.12. GFP Expression in Day 8 pEGIP-iPS-Derived Neurospheres.

GFP expression was observed ubiquitously at low levels in iPS-derived neurospheres (phase contrast A, flourescence B). GFP expression could not be detected by eye in terminally differentiated neurons *in vitro* nor *in vivo* after transplantation of GFP-iPS-derived neural progenitors cells (data not shown).

Non-Viral Constitutive Expression Vector Design

Due to the failure of the previous attempts to create stable expressing cells that express in the neuronal phase and *in vivo,* we tested an additional expression backbone system that had previously been used with success in embryonic stem cells. **Figure 7.13** shows design of GFP, mCherry, GFP-FAK, and mCherry-FAK expression constructs. The Emory University Cloning Core created the clones described here.

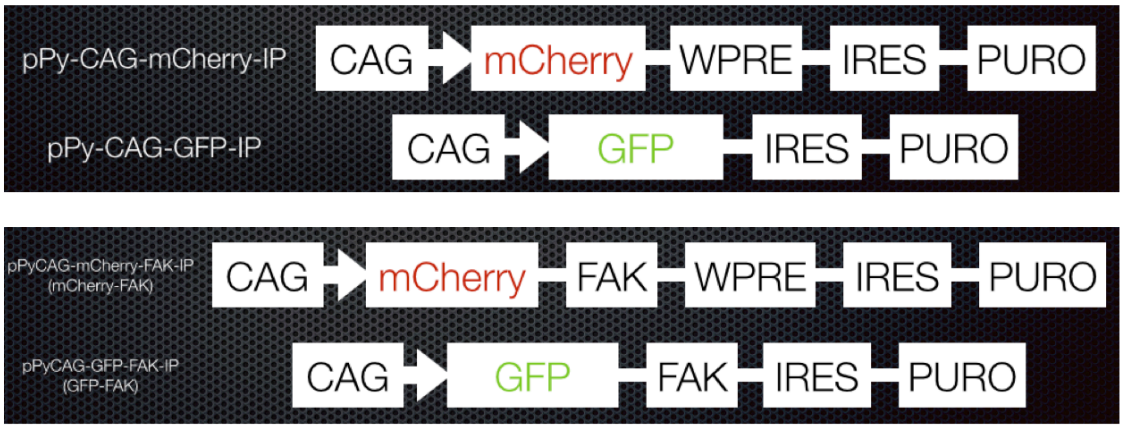


Figure 7.13. Constitutive Expression Vector Design.

GFP, GFP-FAK, mCherry, and mCherry-FAK constitutive expression vectors were designed according to the block diagrams above. Cloning was performed by the Emory University Cloning Core. GFP, mCherry, and FAK (NM_013081.1) coding sequences were subcloned into the region containing the Nanog coding sequence of Addgene Plasmid 13838. These vectors and the resulting cells expressing them will be referred to from here forth as: GFP, mCherry, GFP-FAK, and mCherry-FAK.

Non-Viral Stable Cell Creation

Successful stable transgene expressing cell lines were obtained with the expression vectors described above following transfection of pluripotent colonies with clones and selection with puromycin (**Figure 7.14**). Stable expressing clones were expanded and pooled for each respective cell line. Transgene expression persisted throughout the neurosphere stage (**Figure 7.15.A**) and through DIV 9 in iPS-neurons *in vitro* (**Figure 7.15.C**). mCherry-iPS-NPCs were transplanted *in vivo* and mCherry-expressing cells are observed in coronal sections 28 days after transplantation (**Figure 7.16**). Transplanted mCherry-iPS-NPCs terminally differentiate into neurons expressing NeuN and neurofilament *in vivo* (**Figure 7.17**), demonstrating the utility of the stable mCherry-iPS cells created for cell tracking *in vivo*. Results description and proceeding **Figures 7.14-7.17** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

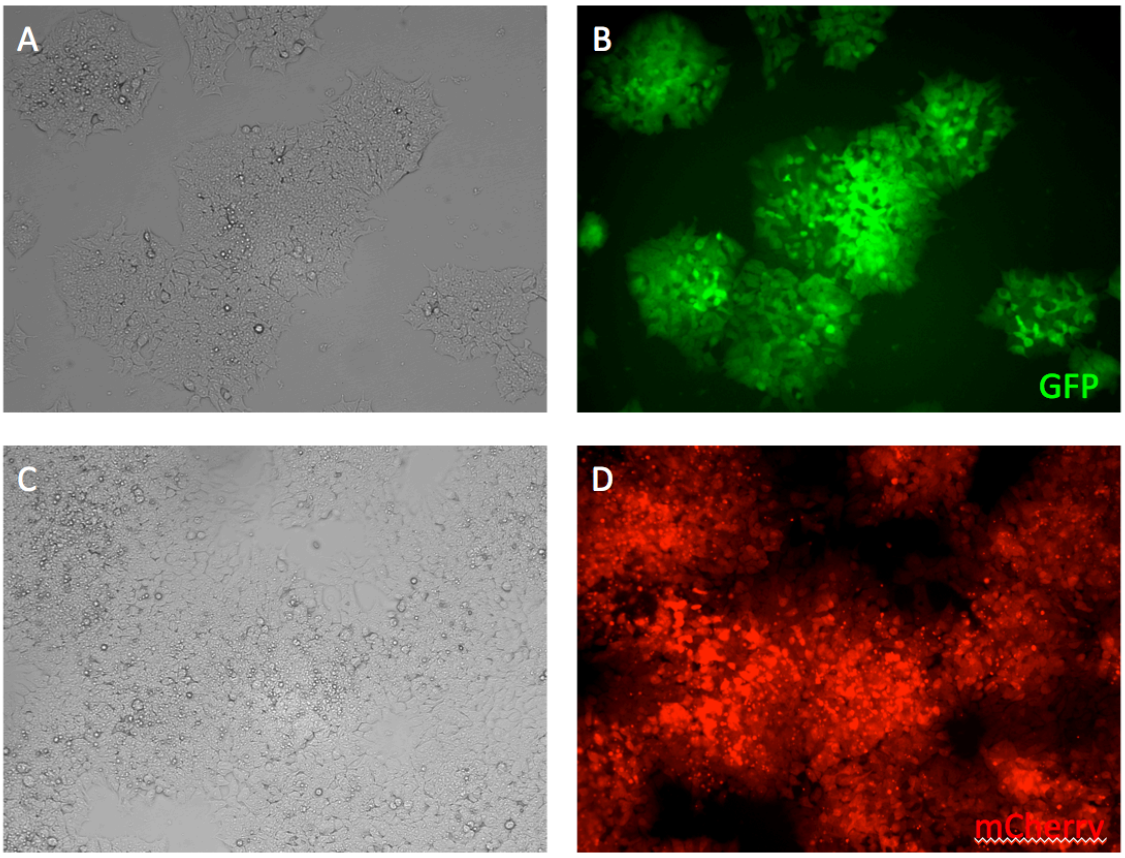


Figure 7.14. Stable GFP and mCherry iPS Pluripotent Colonies.

Pluripotent mouse iPS colonies were transfected with lipofectamine and the GFP and mCherry expression constructs outlined in the previous figure. Stable colony selection was performed with puromycin. Colonies were then pooled and expanded. High GFP and mCherry levels are observed in live cells (Phase contrast A, C; B, D show GFP and mCherry fluorescence).

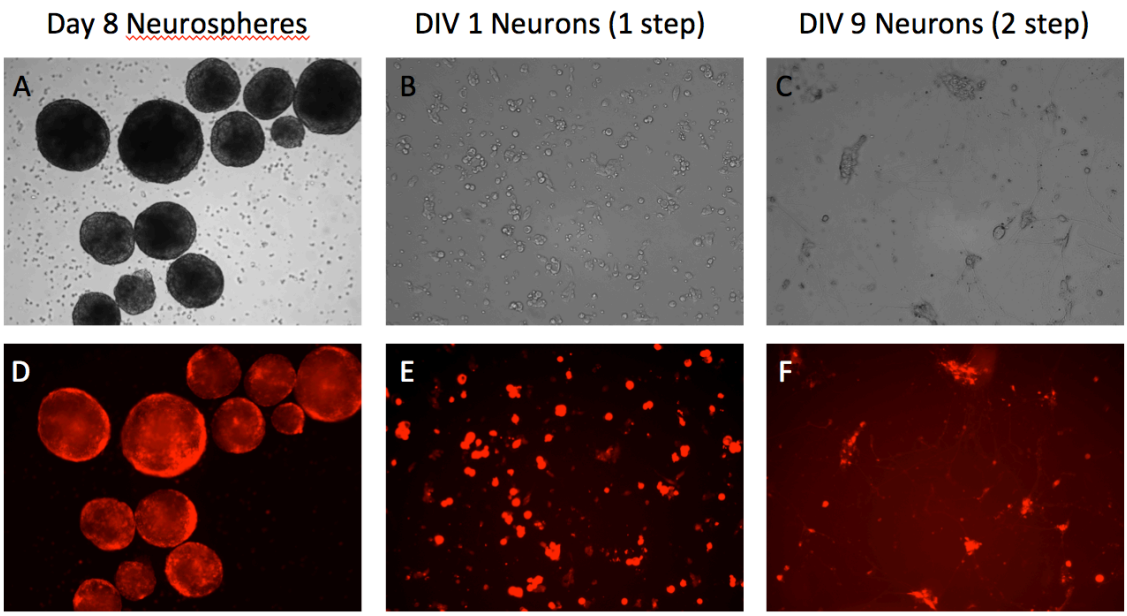


Figure 7.15. mCherry Expression in Stable mCherry-iPSCs.

High levels of mCherry expression are seen in mCherry iPSCs in day 8 neurospheres (A, D), DIV 1 neurons (B, E), and DIV 9 neurons (C, E). These data demonstrate that high transgene expression is maintained throughout all stages of differentiation.

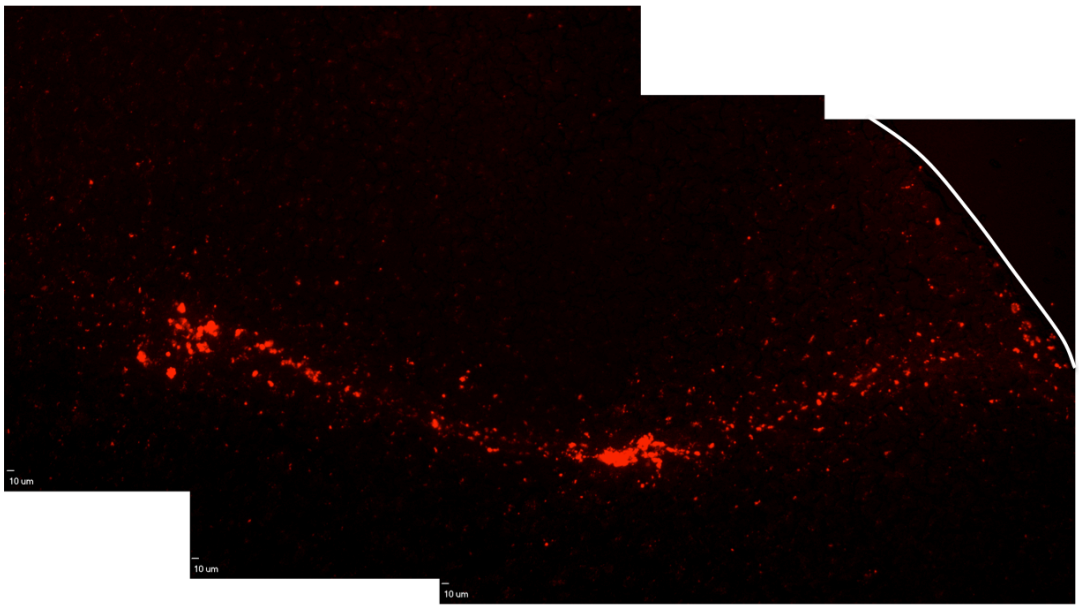


Figure 7.16. mCherry iPS-NPCs and Neurons Can Be Tracked *in Vivo.*

Composite image of a coronal brain section 28 days after intracranial mCherry iPS-NPC transplantation in a normal mouse without stroke. Cells were transplanted into the right hemisphere of a normal animal. mCherry positive cells can clearly be visualized even 28 days after transplantation. The white line denotes the cortical boundary.

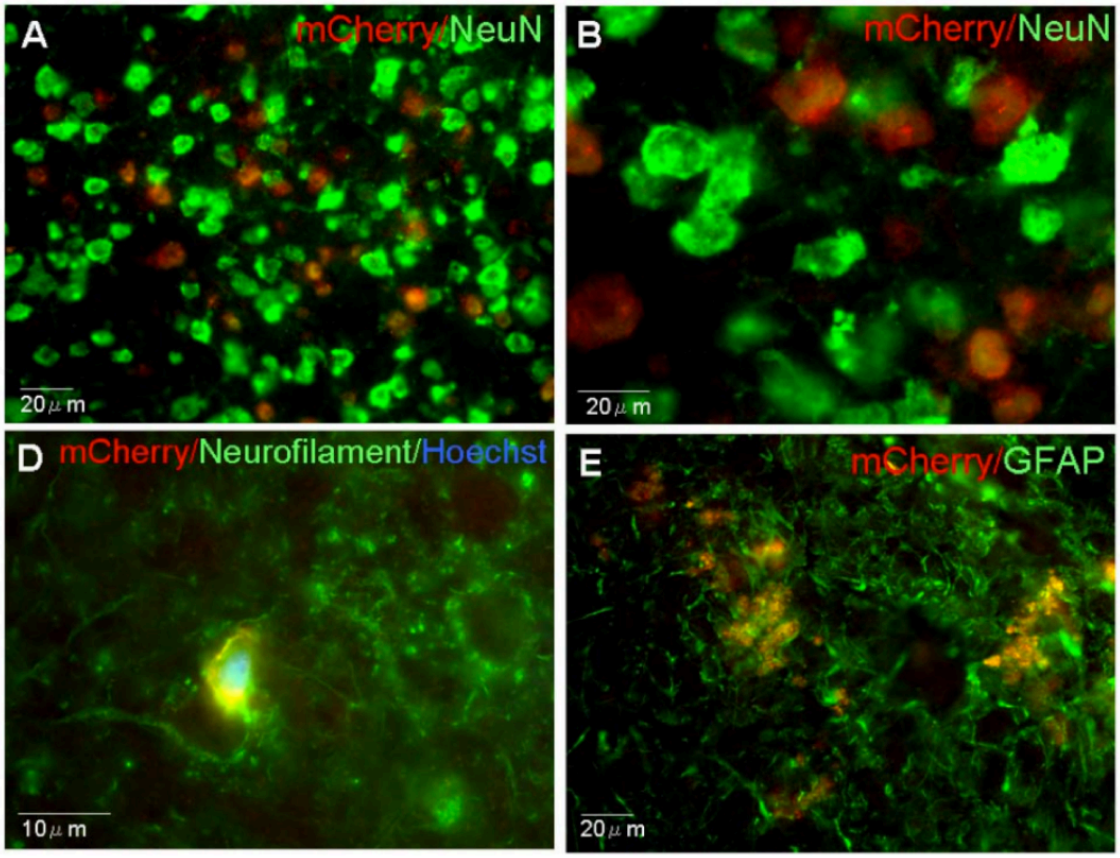


Figure 7.17. Transplanted iPSC-NPCs Differentiate into Neural Lineage Cells.

Transplanted mCherry iPSC-NPCs differentiated into NeuN-positive cells (A,B), neurofilament-positive cells (C), and GFAP-positive cells (D). Republished and adapted with permission from: *iPSC Transplantation Increases Regeneration and Functional Recovery After Ischemic Stroke in Neonatal Rats*, Chau MJ et al., Stem Cells Volume 32(12) pp.3075-3087. Copyright 2014 AlphaMed Press.

FAK-iPS Cell Creation

Following validating of the pPyCAG backbone, the coding sequences for GFP-FAK and mCherry-FAK were subcloned into the backbone by the Emory University Cloning Core. Stable mCherry-FAK and GFP-FAK expressing pluripotent mouse iPS colonies were created with transfection of these clones and puromycin selection (**Figure 7.18.A** and **7.18.B**, respectively). 100% of cells express the fusion transgenes. Massive FAK overexpression is observed in pluripotent mCherry-FAK-iPS colonies (**Figure 7.19**). FAK phosphorylation at the Tyr 397 and Tyr 576/577 positions is increased intensively as well. Immunoblot data at the neurosphere stage is less clear, although FAK overexpression is apparent. There is a lack of Phospho-FAK signal at this stage. In GFP-FAK iPS-NPCs, IHC shows a large GFP-FAK signal in the cell soma and processes at DIV 1 (**Figure 7.20**). Immunoblot data in GFP-FAK neurons shows that FAK overexpression persists through at least DIV 8 (~2.5 times control, **Figure 7.21.A**) and there is a corresponding increase in FAK^{Y397P} levels as well (**Figure 7.21.B**). Similarly, strong mCherry-FAK expression is observed in live iPS-NPCs at DIV 2 (**Figure 7.22**). A large portion of mCherry-iPS, mCherry-FAK-iPS, and GFP-FAK-iPS neurons express NeuN, suggesting that neuronal differentiation is not impaired (**Figure 7.23**). Finally, mCherry-iPS, mCherry-FAK-iPS, and GFP-FAK-iPS neurons all fire action potentials (**Figure 7.24.A**, **7.24.B**, and **7.24.C** respectively), demonstrating that all stable cell lines create functional neurons. Results description and proceeding **Figures 7.18-7.24** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK

is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

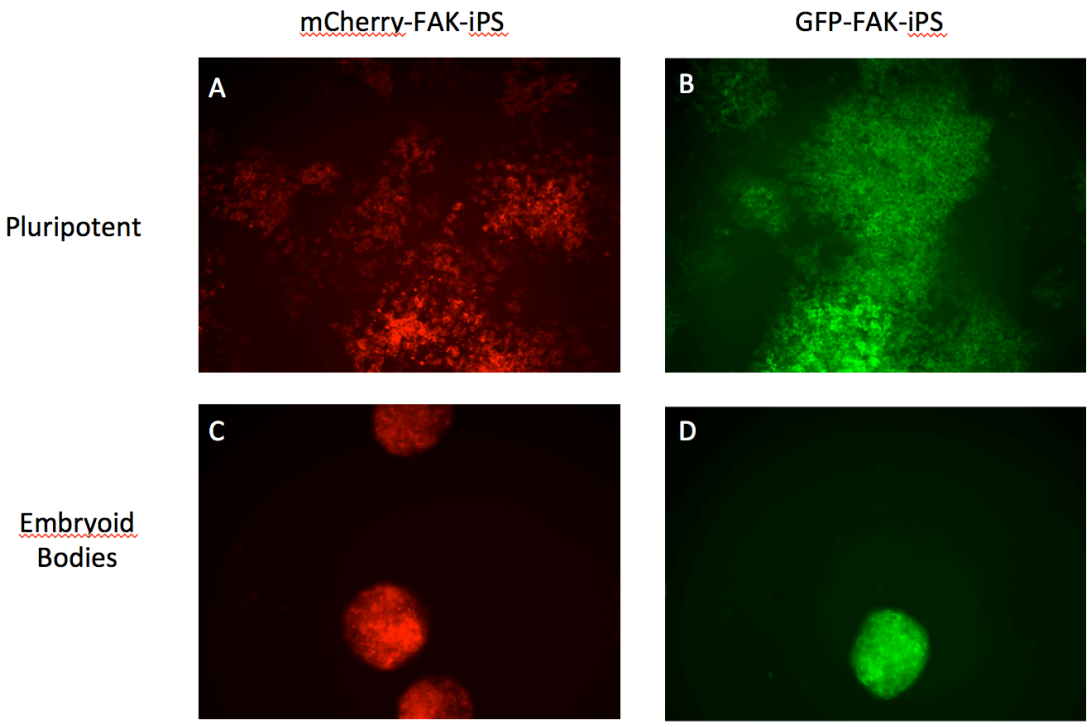


Figure 7.18. Successful Creation of mCherry-FAK and GFP-FAK Stable Mouse iPS Colonies.

Fluorescent images show mCherry-FAK and GFP-FAK expression in live cells. High expression is observed in 100% of cells at the pluripotent and embryoid body (EB) stage.

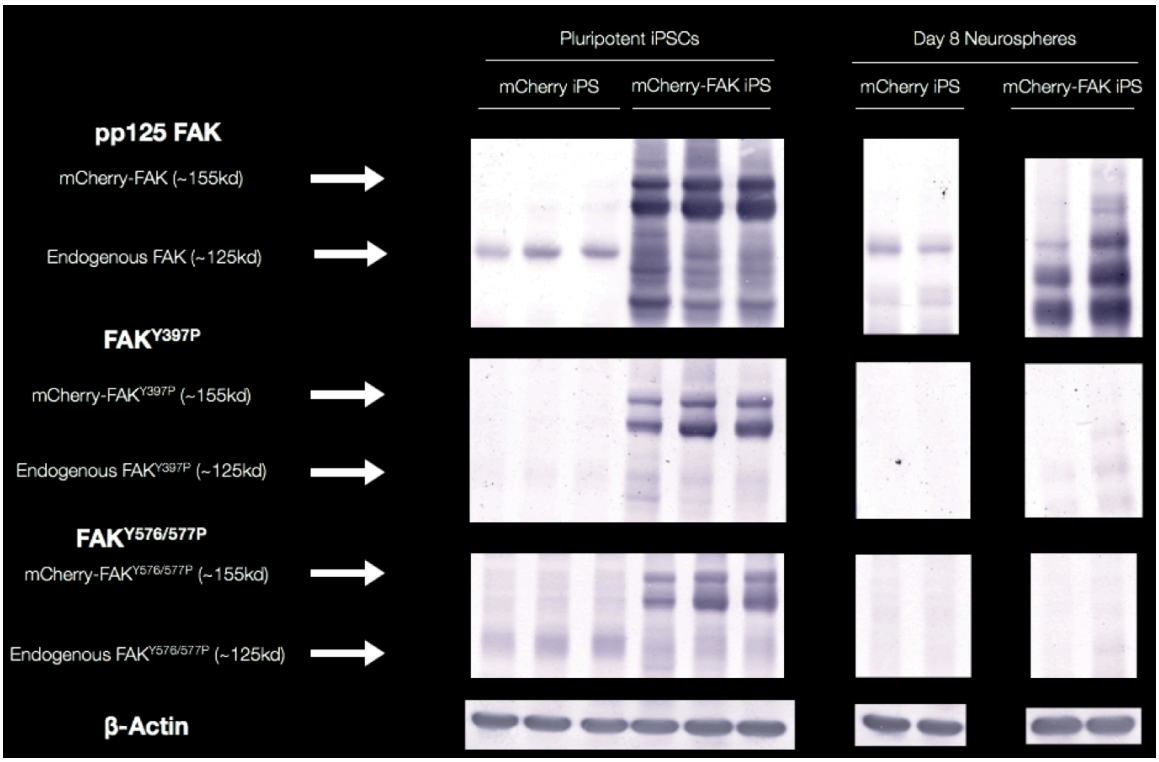


Figure 7.19. FAK Levels in mCherry and mCherry-FAK Stable Cells.

Immunoblots demonstrate massive upregulation of FAK in mCherry-FAK iPS cells compared to mCherry iPS cells at the pluripotent and neurosphere stage. Each lane is one sample pooled in triplicate. Corresponding increases in FAK^{Y397P} and $FAK^{Y576/577P}$ are observed as well. Differences in FAK phosphorylation are most apparent in the pluripotent stage and less apparent in the neurosphere stage.

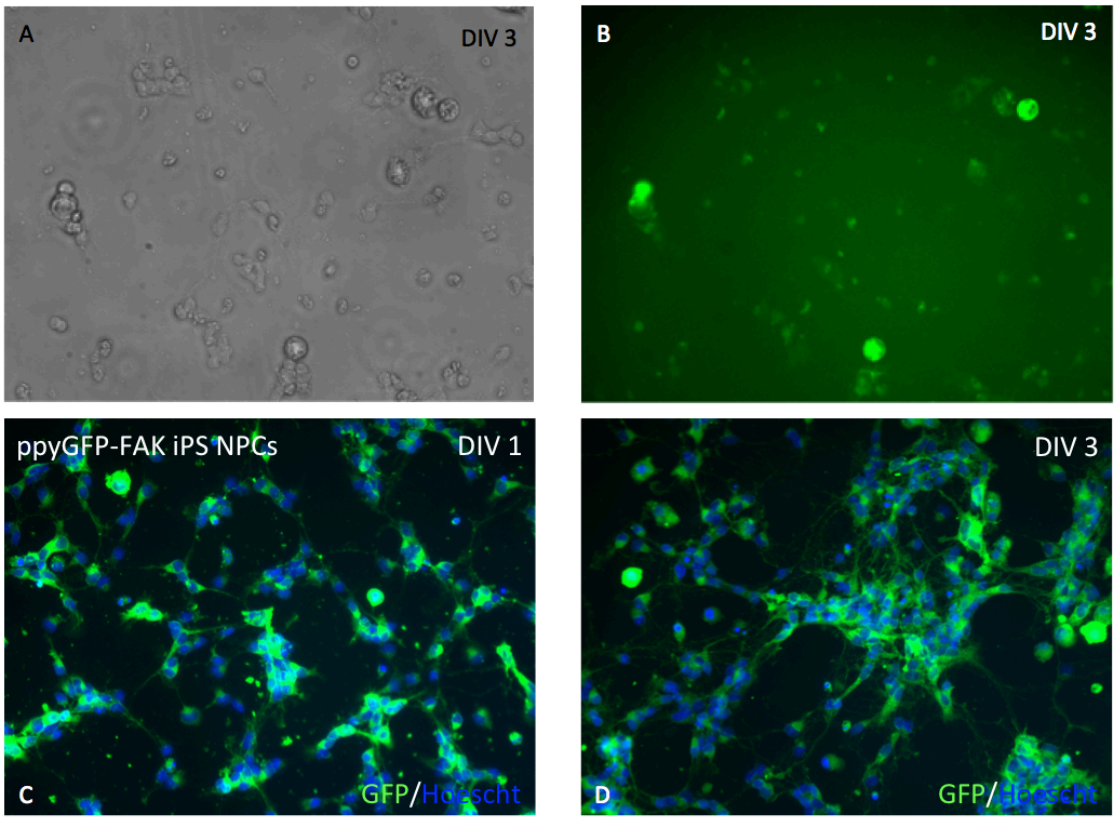


Figure 7.20. GFP-FAK Expression and Localization in DIV-3 iPS-NPCs.

Live cell imaging shows GFP-FAK expression in DIV 3 GFP-FAK iPS-NPCs (A=phase contrast, B). Immunostaining for GFP reveals strong expression at DIV 1 and DIV 3 in the cell cytoplasm and processes (C, D respectively).

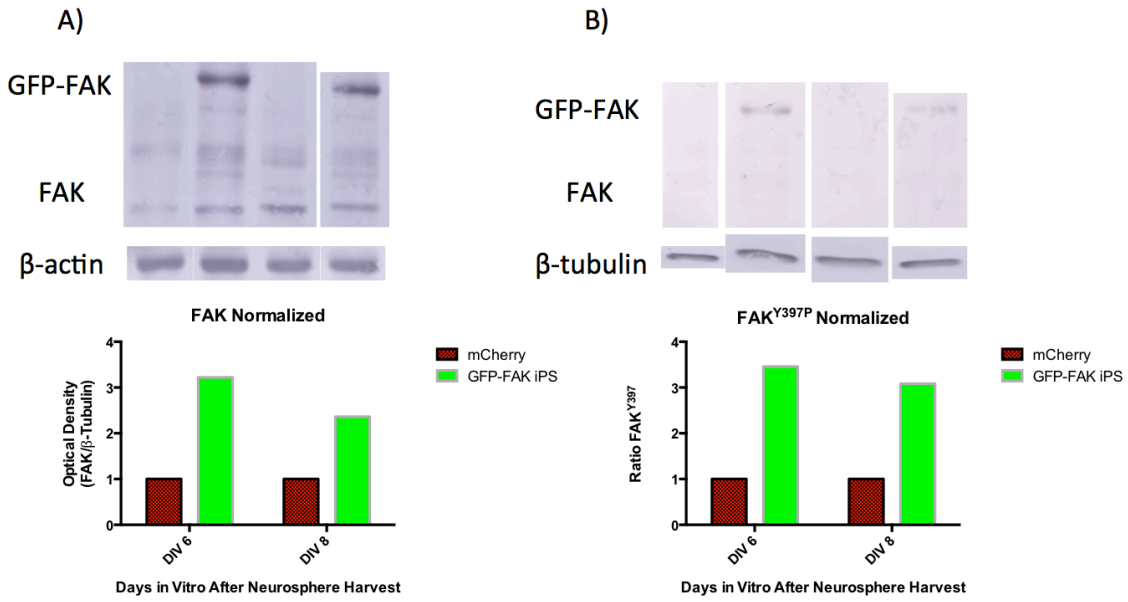


Figure 7.21. FAK Overexpression in GFP-FAK-Derived Neurons.

Western blots from DIV 6 and DIV 8 mCherry and GFP-FAK neurons show a clear upregulation of FAK at both DIV 6 and DIV 8. Endogenous FAK is visualized at 125kd and the GFP-FAK fusion protein is visualized at 152kd. Additionally, phosphorylation at Tyr397 is increased, demonstrating that FAK is not only being overexpressed, but it is active as well. Immunoblots in (B) show that most of the phosphorylated FAK is in the band of higher molecular weight, demonstrating that the majority of phosph-FAK is from the exogenous expression vector.

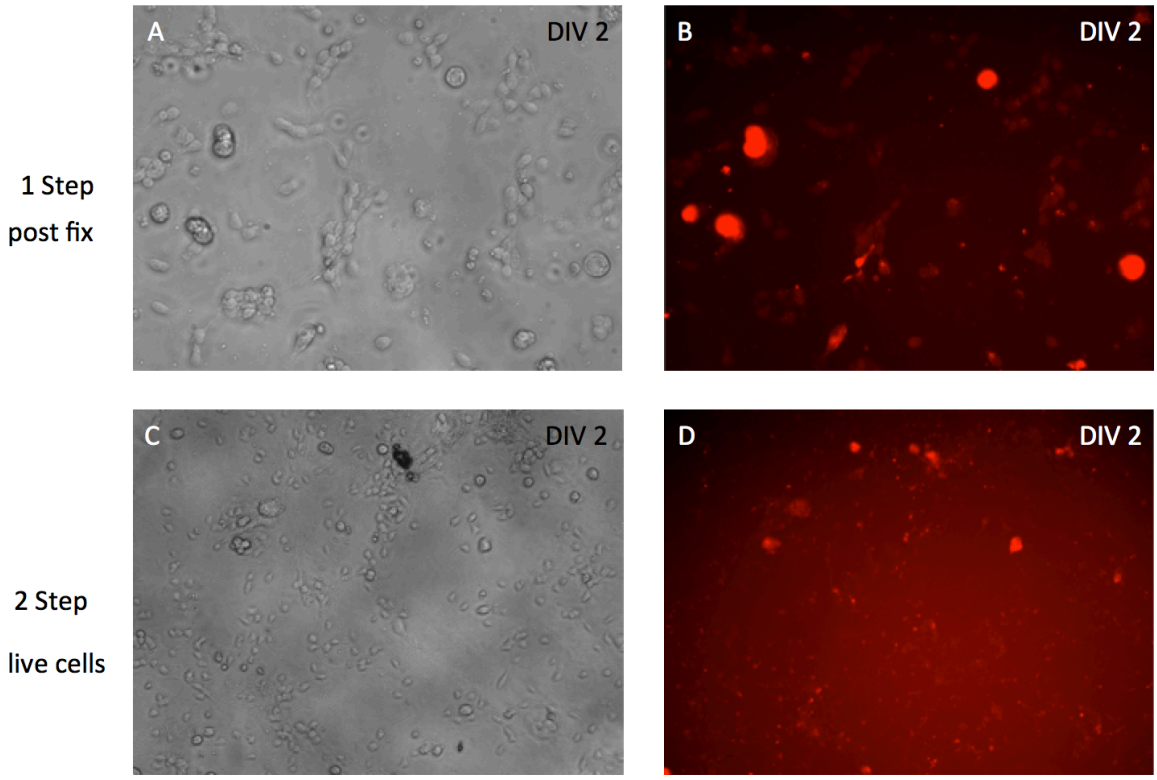


Figure 7.22. mCherry-FAK Expression in Live mCherry-FAK iPS-NPCs.

Live cell imaging shows mCherry-FAK expression in DIV 2 mCherry-FAK iPS-NPCs. In both “one-step” and “two-step” culture, cells show strong mCherry-FAK signal (A, C = phase contrast; B, D = flourescent).

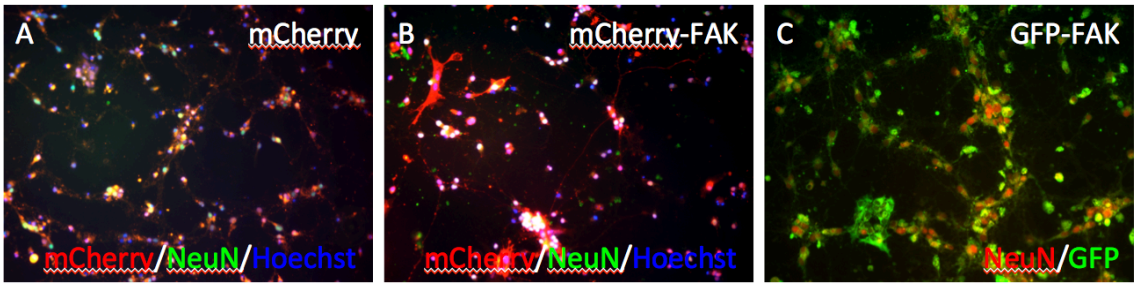


Figure 7.23. Stable Transgenic FAK Neurons Express NeuN.

High percentages of cells express NeuN in DIV 8 mCherry iPS-neurons (A), mCherry-FAK iPS-neurons (B), and GFP-FAK iPS-neurons (C).

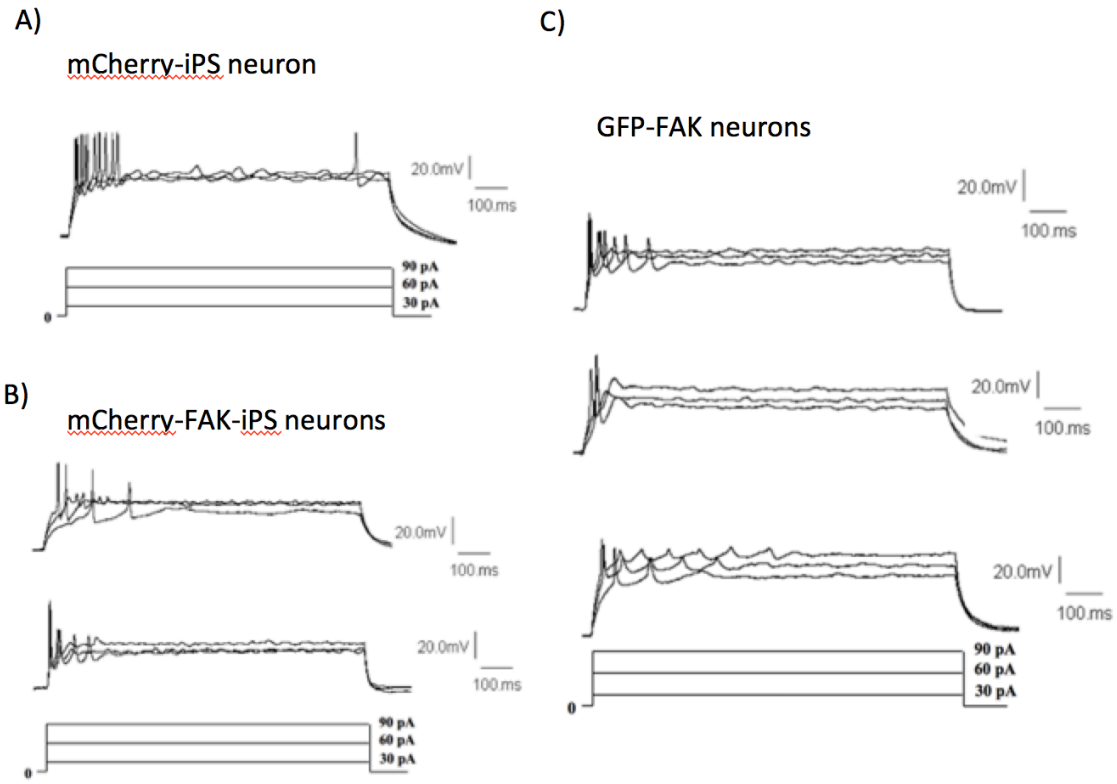


Figure 7.24. Stable Transgenic iPS-Derived Neurons Fire Action Potentials.

Whole-cell electrophysiology was performed on DIV 10 mCherry, mCherry-FAK, and GFP-FAK iPS-derived neurons in current clamp mode, stepping in + 30pA increments from 30pA to 90pA. Representative traces from mCherry (A), mCherry-FAK (B), and GFP-FAK (C) show that neurons fire action potentials.

Discussion

The creation of transgenic iPS cells serves a dual purpose. First, creating stable cell lines that express fluorescent transgenes such as GFP provide a reliable means to track transplanted cells *in vivo*. Reliable tracking of cells is necessary to further iPS transplantation research and to help answer questions relating to terminal cell fate and integration into host circuitry. Second, genetic modifications of iPS cells in terms of overexpression or gene silencing may enhance physiological aspects related to survival, migration, integration, etc. These cellular modifications before transplantation may improve transplantation and iPS graft efficacy *in vivo*.

One of the concerns of iPS transplantation for any bodily disorder is the potential for tumorigenesis after transplantation (Kawai, Yamashita et al. 2010). iPS cells by definition are pluripotent and as a result, transplantation of undifferentiated iPS cells has a large potential for teratoma formation. Although we transplant differentiated iPS cells and have not observed any adverse effects, the risk is still there. Genetic modifications of iPS cells before transplant may be beneficial for transplant, however due to the risk of teratoma formation, aberrant expression may aid teratoma formation or metastasis of said tumors. Especially concerning FAK, which has been shown to be upregulated in metastatic and aggressive tumors, constitutive expression may be detrimental in the long run. Doxycycline-inducible expression systems (or other inducible expression systems) provide a means to control transgene expression with a drug "switch". We attempted to take this route when we first tried stable cell creation and FAK

overexpression. We were not successful. Although we were not successful, technical hurdles should not preclude the utility of this approach, and it is worth considering in future studies.

A challenge in creating transgenic iPS cells compared to other mammalian cell types is that transgene must be maintained in different cellular "states": pluripotent, differentiated, and terminally differentiated. Following the failure of our inducible approach, we succeeded in creating stable expressing iPS colonies where 100% of cells expressed transgene. Additionally, pluripotent marker expression was maintained in stable colonies, suggesting that the selection process and transgene expression did not interfere with pluripotency. Expression of GFP in these was maintained up to the neurosphere stage, however we observed a sharp drop in expression following neurosphere dissociation and terminal differentiating. The cause of this is currently unexplored, however we suspect an epigenetic-like gene silencing is occurring in the transgene coding sequence that is introduced into the genome by the lentivirus.

Following the previous failures, we decided to take a different approach and tested a unique episomal self-replicating backbone that has been previously described in embryonic stem cells (Gassmann, Donoho et al. 1995, Camenisch, Gruber et al. 1996, Mitsui, Tokuzawa et al. 2003). Ostensibly, this vector should maintain high expression in all stages of culture due to self-replication, and additionally should be immune to epigenetic gene silencing because it maintains itself outside of chromosomal DNA.

GFP, mCherry, GFP-FAK, and mCherry-FAK coding sequences were cloned into this backbone. These clones were then transfected into pluripotent mouse iPS colonies and stable selection was peformed with puromycin. We observed that 100% of cells expressed the transgenes, and expression was maintained at high levels in the pluripotent stage, embryoid body/neurosphere stage, and neural progenitor/neuron stage. Additionally, transgene expression in transplanted cells was seen *in vivo* at least 28 days after transplantation. These cells differentiated into cells that express NeuN at high efficiency and fire action potentials *in vitro*, suggesting that transgene expression and stable cell selection did not interfere with differentiation.

FAK is ubiquitously expressed at high levels in mammalian cells. In order to create a cell line that overexpresses FAK and has an observable phenotype, we hypothesized FAK expression would need to be at least 2-3 times normal physiological levels in iPS-NPCs. Immunoblots from pluripotent iPS colonies show massive FAK overexpression and a dramatic increase in Phospho-FAK in mCherry-FAK-iPS cells, however the differences are difficult to quantify due to intermediate products of the fusion protein. mCherry-FAK expression is observed at high levels in live cells in DIV 2 iPS-NPCs, further demonstrating that mCherry-FAK expression is present at this time point. IHC with GFP-FAK iPS-NPCs also demonstrates that GFP-FAK is present at high levels after terminal differentiation. Immunoblots of GFP-FAK neurons at DIV 6 and DIV 8 demonstrate that FAK expression is 2-3 times normal expression in terms of total FAK and FAK^{Y397P}. Taken together, these data show we accomplished our goal of creating stable FAK overexpressing cells that can

be traced *in vivo*. Addtionally, FAK upregulation is to a degree that should generate a noticeable phenotype. From this point forward, we chose to go with cell lines expressing the mCherry flourophore; it is easier to detect *in vivo* because its observed fluorescence is higher than GFP and animals receiving mCherry-labeled cells do not require perfusion for tissue processing and staining.

Chapter 8 - Effect of FAK Overexpression on iPSC Attachment, Migration, and Neurite Outgrowth

Introduction

We have demonstrated in the previous chapters that iPS cells can be efficiently differentiated into neurons, and that these cells express high levels of FAK and other pro-migratory proteins. We also demonstrated successful efficient generation of stable iPS cells lines where 100% of cells therein express a transgene of interest. We created several FAK-overexpressing iPS cell lines that overexpress FAK at least 2-3 times normal physiological levels and that the FAK expressed by the exogenous expression construct is phosphorylated and active.

Current hurdles to optimal transplantation studies include low cell survival, low cell migration, and unknown levels of integration into host circuitry. FAK has been shown to affect cell attachment (Hungerford, Compton et al. 1996), cell survival (Frisch, Vuori et al. 1996, Hungerford, Compton et al. 1996), cell migration (Owen, Ruest et al. 1999, Reiske, Kao et al. 1999, Wang, Park et al. 2000, Xie and Tsai 2004, Lee, Lee et al. 2010, Lee, Lee et al. 2010, Yun, Ryu et al. 2010), and neurite outgrowth (Li, Lee et al. 2004, Ren, Ming et al. 2004, Moore, Zhang et al. 2012), making it an ideal candidate for manipulation in iPS cells prior to transplantation. In the following chapter, we investigated the role of FAK in these physiological properties in iPS-NPCs with several *in vitro* assays.

Additionally, previous chapters have mentioned that genetic manipulation of iPS cells is only one of several strategies that may be used to improve cell survival, attachment, and migration after transplantation. We have previously demonstrated in other cell types that hypoxic preconditioning can improve survival and migration

of MSCs (Hu, Wei et al. 2011, Sun, Wei et al. 2015, Wei, Gu et al. 2015). We employ a similar strategy here. Last, we also demonstrate that iPS migration can be increased with treatment of proteins, such as WNT-3a.

Results

Oxygen-Glucose Deprivation

Cell transplantation in rodent models of ischemic stroke occurs within 7 days of stroke, within which time reperfusion of the ischemic brain has not reached 100% of pre-stroke levels. Transplanted cells therefore experience an ischemic environment, to a degree, which can be detrimental to transplanted cell survival. Several studies have linked decreases in FAK signaling to decreased cell survival following ischemia (Zalewska, Makarewicz et al. 2005, Zalewska, Ziemka-Nałęcz et al. 2005, Hakim, DiMichele et al. 2009). Increasing FAK signaling may therefore improve cell survival following ischemia. To simulate an ischemic environment *in vitro*, DIV 6 iPS-neurons were subjected to oxygen-glucose deprivation (OGD) for 4 hours. Cell viability was assayed with a common MTT assay. Cells were subjected to 4 hours of OGD followed by 24 hours of reperfusion, which was simulated by addition of normal glucose levels and culture in normoxic conditions. 4 hours OGD reduced cell viability to ~50% of no-OGD control. Two experiments were performed in triplicate. No differences in cell viability were observed between mCherry and mCherry-FAK cells (**Figure 8.1**). Results description and proceeding

Figure 8.1 are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

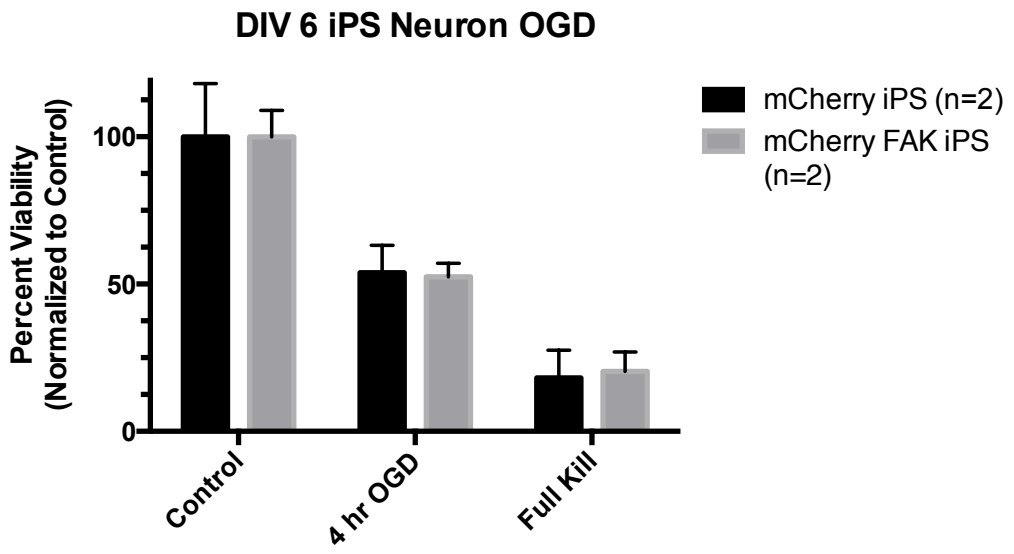


Figure 8.1. Oxygen-Glucose Deprivation.

DIV 6 mCherry and mCherry-FAK iPS-derived neurons were subjected to oxygen-glucose deprivation. Cell viability was measured with an MTT assay. 4 hour OGD decreases cell viability to ~50% of control. No differences were observed between mCherry and mCherry-FAK cells.

iPS-NPC Attachment

We examined cell attachment by harvesting and dissociated neuropheres; plating the resulting cells (300,000 cells/dish); washing, fixing, and counterstaining cells with Hoechst; and counting Hoechst-positive cells. There was a slight increase observed in mCherry-FAK cell attachment compared to mCherry-iPS cells, however this was non-significant (**Figure 8.2.A, 8.2.B**). Addition of the FAK inhibitor, FAK-14, significantly decreased attachment of mCherry and mCherry-FAK iPS-NPCs (**Figure 8.2.A, 8.2.B** , n=3/group in triplicate, two-way ANOVA $F(1,8) = 21.01$, p=0. 0.0342 and p=0.0166 respectively). Results description and proceeding **Figure 8.2** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

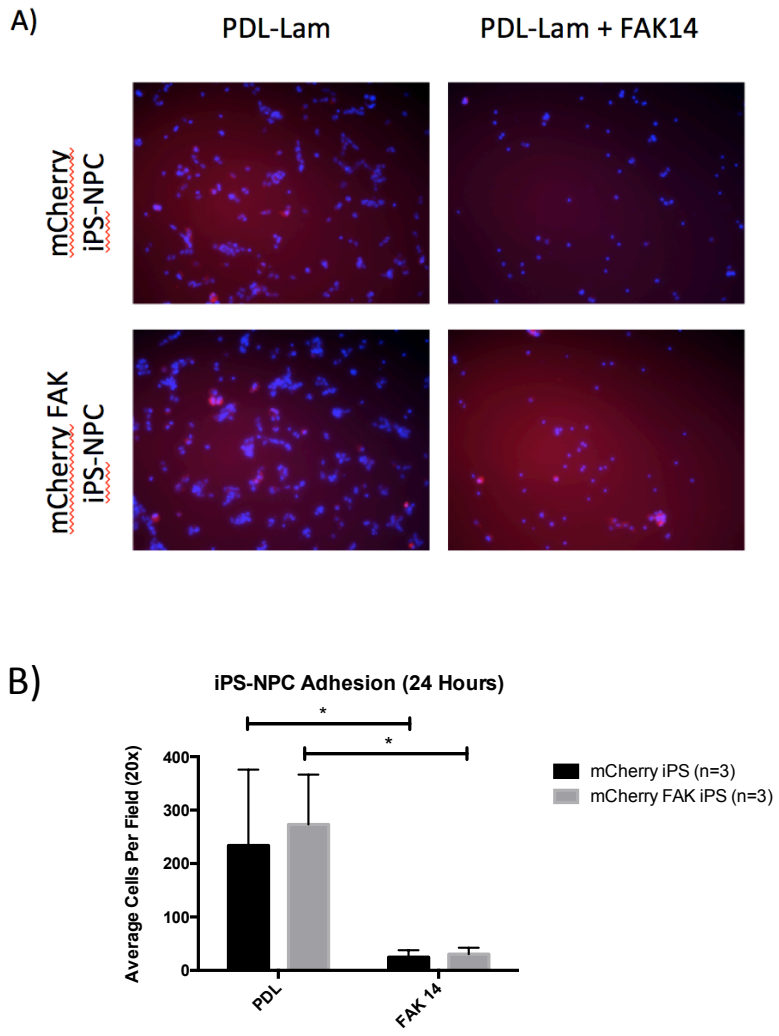


Figure 8.2. iPS-NPC Attachment.

After neurosphere harvest and dissociation, DIV 0 mCherry and mCherry-FAK iPS-derived neurons were plated on PDL-laminin coated 35mm dishes. 24 hours later, cells were fixed and counted to determine differences in attachment to the PDL-laminin substrate. The specific FAK inhibitor FAK 14 was added (10μM) to a subset of dishes to determine the effect of inhibition in addition to upregulation. mCherry-FAK cells showed a non-significant trend in increased attachment in the raw and

normalized data (A, B, n=3). FAK-14 significantly decreased attachment in mCherry iPS cells and in mCherry-FAK iPS cells (B, two-way ANOVA $F(1,8) = 21.01$, $p=0.0342$ and $p=0.0166$ respectively).

iPS-NPC Migration

A common trans-well migration assay was used to assess iPS-NPC migration. After harvest and dissociation, DIV 0 mCherry and mCherry-FAK iPS-NPCs (200,000/chamber) were plated onto Matrigel® coated transwell inserts. Cells were allowed to migrate through the coated inserts in the presence of one of three chemoattractive "gradients": SATO media only (no SDF-1α), SATO media + SDF-1α (200ng/μL), or SATO media + SDF-1α (200ng/μL) + FAK-14 (10μM). SDF-1α was selected as the chemoattractant because it is upregulated and secreted by injured neuronal tissue after ischemic stroke. We observed that migration of mCherry and mCherry-FAK iPS-NPCs was significantly increased in the presence of an SDF-1α chemoattractive gradient (**Figure 8.3.A, 8.3.B,** n=3/group in triplicate, two-way ANOVA $F(2,84)=53.93$, $p<0.0001$ and $p<0.0001$ respectively). There was a slight trend in increased migration of mCherry-FAK-iPS-NPCs compared to mCherry-iPS-NPCs, however this trend was not significant. Addition of FAK-14 (10μM) to the SDF-1α gradient inhibited mCherry and mCherry-FAK iPS-NPC migration significantly (**Figure 8.3.A, 8.3.B,** n=3/group in triplicate, two-way ANOVA $F(2,84)=53.93$, $p<0.0001$ and $p<0.0001$ respectively). Results description and proceeding **Figure 8.3** are present in a manuscript currently in preparation (**Deveau TC**, Yu SP, Wei L. FAK is Necessary for Induced Pluripotent-Derived Neural Progenitor Cell Attachment and Migration. *Research article, in preparation*).

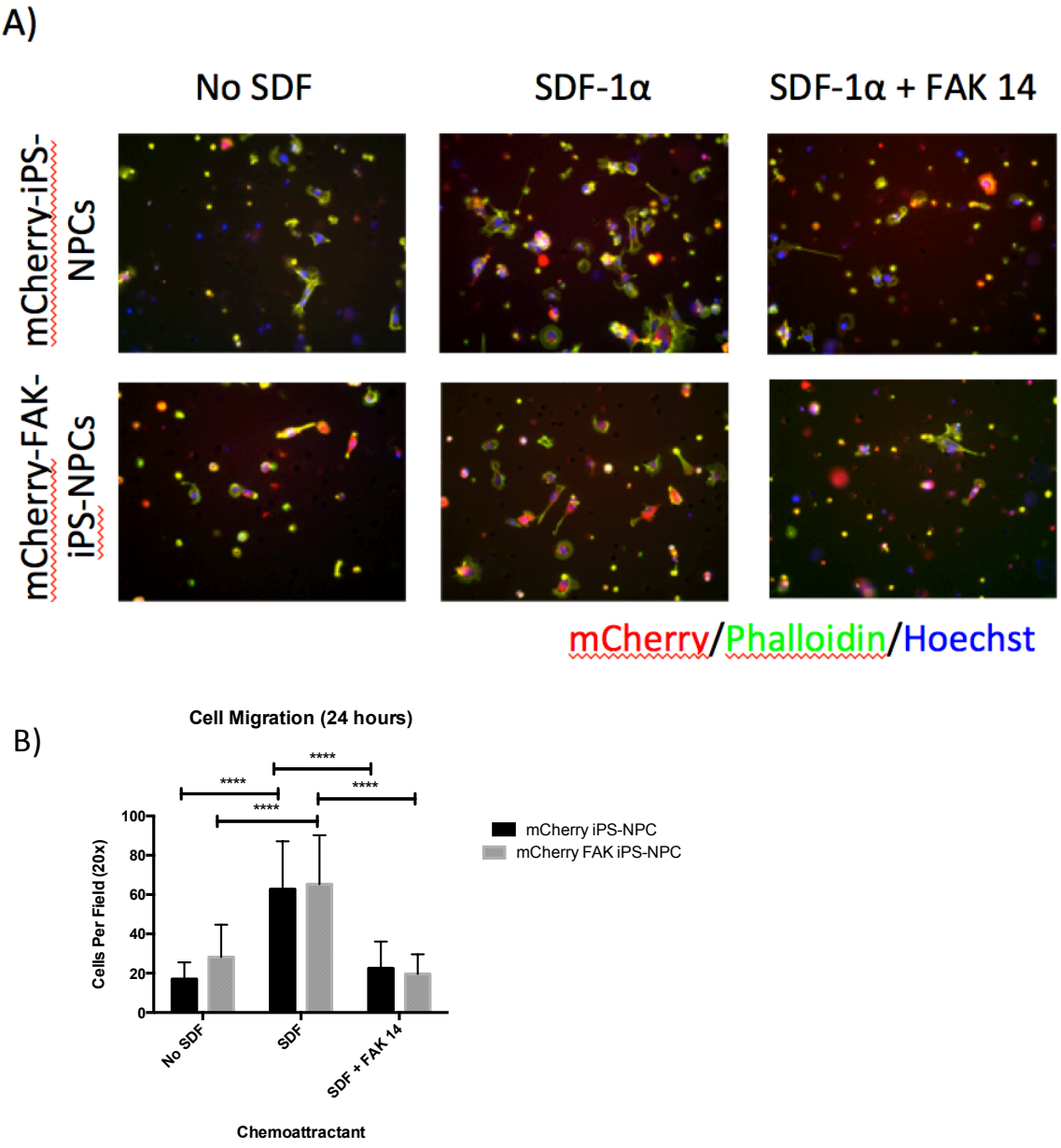


Figure 8.3. iPS-NPC Migration.

After neurosphere harvest and dissociation, DIV 0 mCherry and mCherry-FAK iPS-derived neurons were plated on Matrigel® coated transwell inserts (200,000 cells/insert). Cells were allowed to migrate in either the presence of SATO, SATO +

SDF-1α, or SATO + SDF-1α + FAK 14. mCherry iPS-NPC and mCherry-FAK iPS-NPC migration was significantly increased through the Matrigel® coated inserts in the presence of an SDF-1α chemoattractive gradient (n=3/group, two-way ANOVA $F(2,84)=53.93$, $p<0.0001$ and $p<0.0001$ respectively). FAK-14 addition (10μM) significantly blocked migration in the presence of SDF-1α (n=3/group, two-way ANOVA $F(2,84)=53.93$, $p<0.0001$ and $p<0.0001$ respectively).

mCherry-FAK iPS Neurospheres Show Improved Neurite Outgrowth

Day 8 iPS-derived neurospheres from mCherry and mCherry-FAK cells were harvested and plated on PDL-laminin coated 35mm dishes in SATO media with or without Netrin-1 (200ng/μL). Neurospheres were imaged 48 hours after plating. White arrows denote neurite outgrowth. Without Netrin-1, we observed an increased number of neurites from mCherry-FAK neurospheres compared to mCherry neurospheres that also had increased length. In the presence of Netrin-1, mCherry-FAK neurospheres showed increased neurite outgrowth in terms of number of length compared to mCherry neurospheres. Interestingly, Netrin-1 stimulation did not improve neurite outgrowth in mCherry neurospheres.

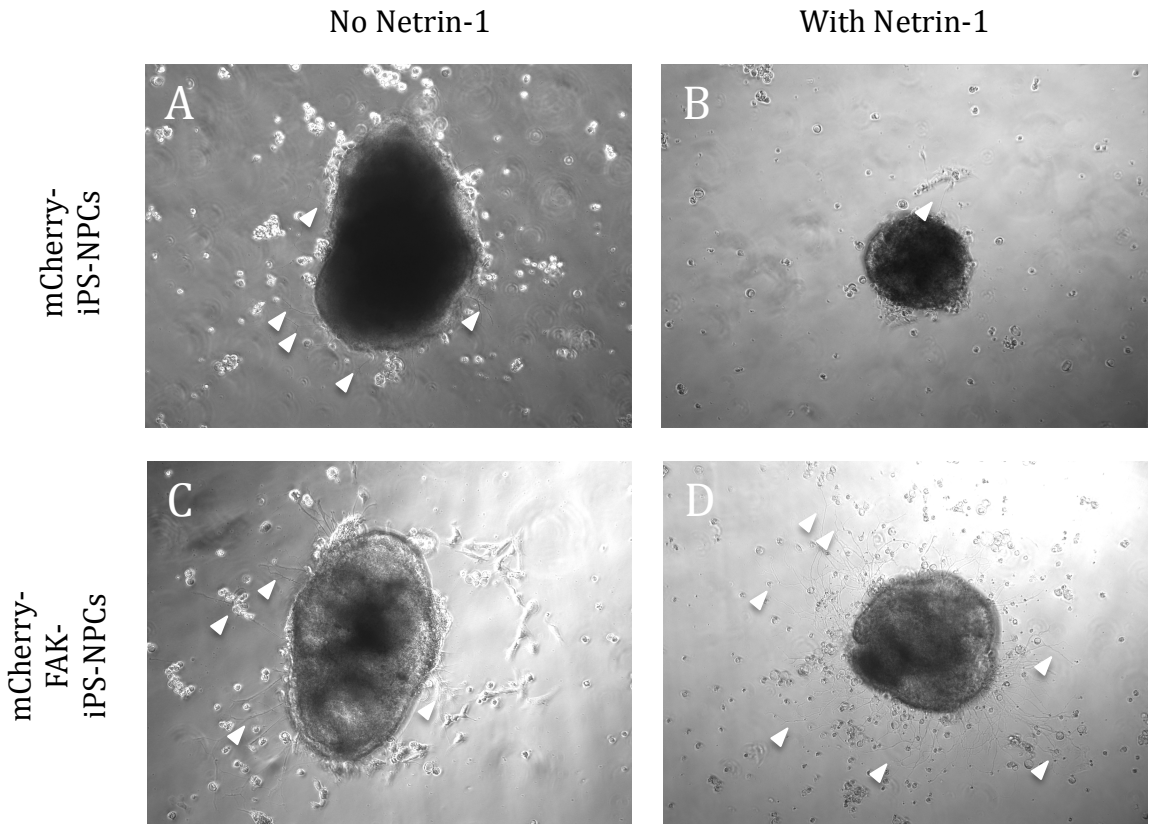


Figure 8.4. iPS-NPC Neurite Outgrowth.

mCherry and mCherry-FAK iPS neurospheres were plated directly onto PDL-laminin coated dishes with (B, D) or without (A,B). Representative neurospheres are shown above. mCherry-FAK neurospheres (B, d) show increased neurite outgrowth in terms of number of neurites and length of neurites compared to mCherry neuropheres. Interestingly, Netrin-1 does not appear to increase neurite outgrowth from mCherry iPS neurospheres (B). As this is an N of 1, further quantitative experimentation is necessary.

Hypoxia Preconditioning Upregulates FAK in MSCs

Hypoxia preconditioning is another mechanism through which migration can be increased and FAK can be upregulated. As a proof-of-principle, we demonstrate PCR data showing FAK upregulation in MSCs after 24 hours of hypoxia preconditioning (in 0.4-0.6% O_2) followed by one hour of reoxygenation. There is a trend in increased FAK expression at the mRNA level in HP groups compared to normoxia groups (**Figure 8.5.A and 8.5.B**, n=7-9/group, *students t-test*, p=0.19).

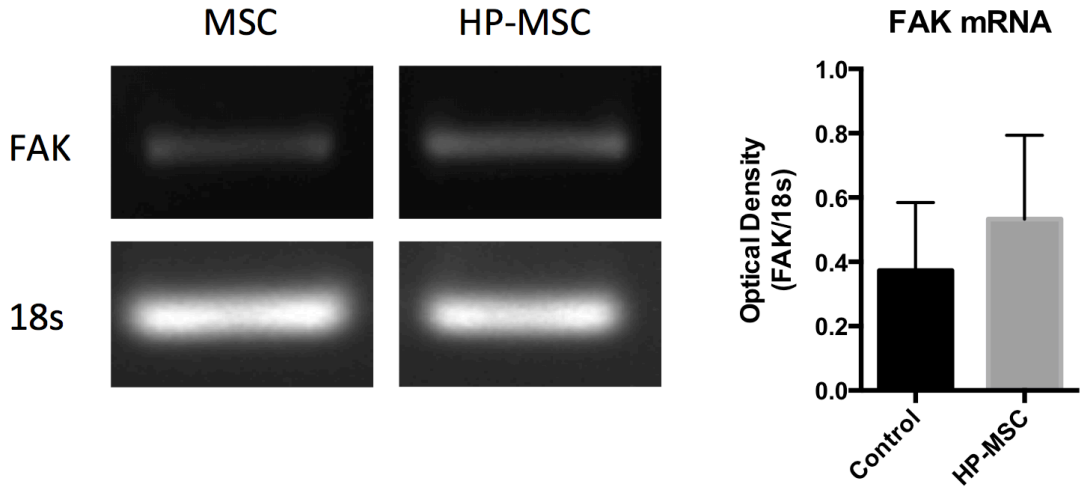


Figure 8.5. Hypoxia Preconditioning Upregulates FAK in MSCs.

Semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) shows a trend in transcriptional upregulation of FAK in HP-MSCs compared to normoxic MSCs (N=7-9/group, p=0.19).

Hypoxia Preconditioning in iPS-NPCs

Because FAK upregulation failed to increase survival, attachment, or migration in our experimental conditions, we examined the effect of hypoxia preconditioning on iPS-NPCs. Day 8 iPS neurospheres were subjected to normoxic conditions, 4-hour hypoxia preconditioning, or 7-hour hypoxia preconditioning (at 0.2% O_2). Cells were then placed in normal culture conditions for one hour and neurospheres were harvested, dissociated, and plated. Before plating, we analyzed cells with a Millipore Scepter™. Hypoxia preconditioning shifted the cell population towards larger diameters and volumes compared to normoxic neurospheres (**Figure 8.6**). Cell shrinkage is a hallmark of apoptotic cell death, so this shift is indicative of a protective effect of hypoxia preconditioning on iPS-NPCs. Hypoxia preconditioning also appeared to decrease cell debris, another hallmark of cell death *in vitro* (**Figure 8.6**). We assayed these cells for cell attachement and cell death. Attachment assays (**Figure 8.7.A, 8.7.B**) demonstrated a decrease in adherent cells in the hypoxia-preconditioned group. Trypan blue staining showed a slight decrease in trypan blue positive cells following 4-hours of hypoxia preconditioning and a slight increase with 7-hour hypoxia preconditioning. Neither of these observations were statistically significant compared to normoxic controls (**Figure 8.7.C**).

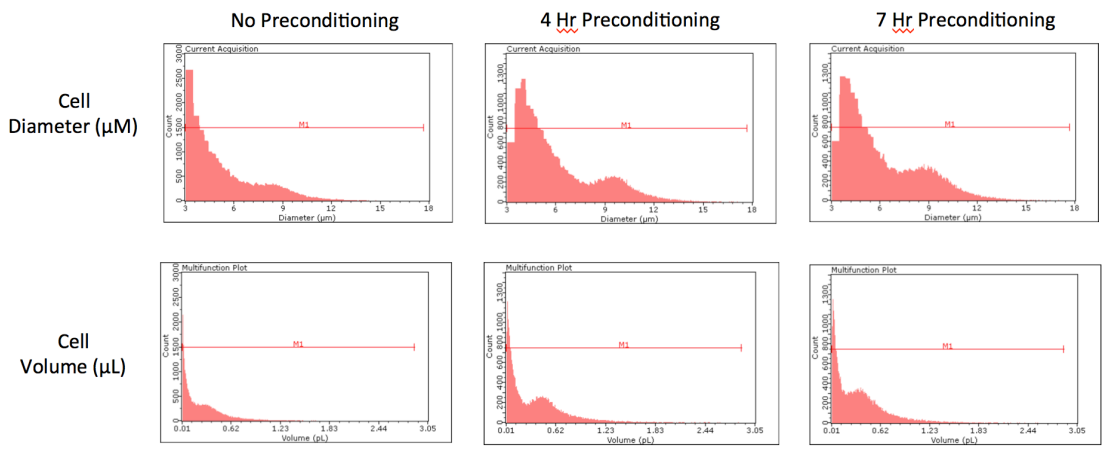


Figure 8.6. iPS-NPC Hypoxia Preconditioning Increases Cell Diameter and Volume.

Neurospheres were subjected to either no preconditioning, 4-hour, or 7-hour hypoxia preconditioning. After an hour of reoxygenation, neurospheres were harvested, dissociated, and analyzed with a Millipore Scepter™. Observations indicate that hypoxia preconditioning increases the fraction of cells with larger cell volumes and diameters.

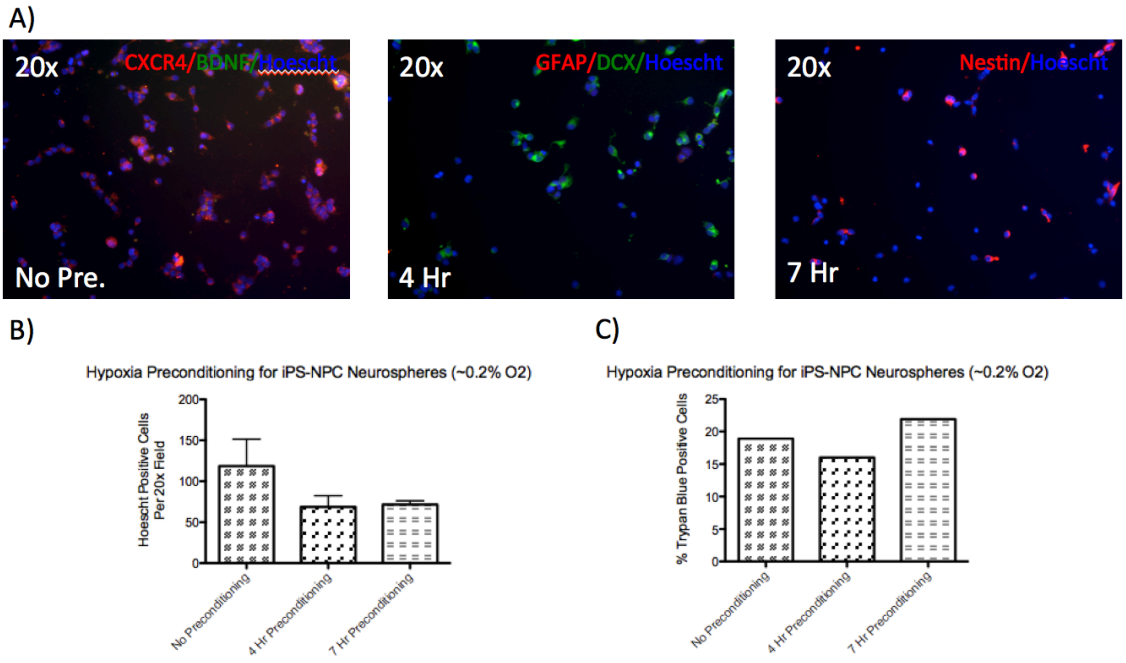


Figure 8.7. Hypoxia Preconditioning Decreases iPS-NPC Attachment.

Neurospheres were subjected to either no preconditioning, 4-hour hypoxia preconditioning, or 7-hour hypoxia preconditioning. After an hour of reoxygenation, neurospheres were harvested, dissociated, and plated. 24 hours later, cells were fixed and the total number of Hoechst positive cells per field analyzed. There was a decrease in cell attachment with 4-hour and 7-hour preconditioning compared to control but the decrease was not statistically significant (A, B; n=3-4, one-way ANOVA $F(2,18)=2.306$, $p=0.2591$ and $p=0.3059$ respectively). We also examined cell death with a standard trypan blue assay 24 hours after plating. There were no significant differences between groups.

WNT-3a Increases iPS-NPC Migration

In addition to genetic manipulation and hypoxia preconditioning, incubation of cells with small molecules, peptides, or proteins before transplantation is another mechanism to improve cell survival, attachment, and/or migration. mCherry iPS-neurospheres were harvested, dissociated, and plated on Matrigel® coated transwell inserts. A subset of cells was plated with WNT-3a to try and stimulate cells and increase migration. SDF-1α was again used as a chemoattractant. Following WNT-3a stimulation, WNT-3a stimulated mCherry iPS-NPCs migrated significantly more through the transwell insert than control mCherry iPS-NPCs (**Figure 8.8.A, 8.8.B**, n=2/group in triplicate, *student's t-test,* p=0.0137).

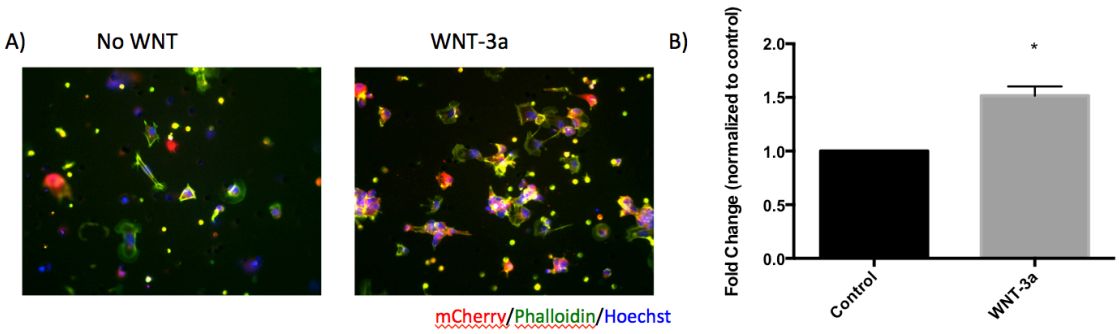


Figure 8.8. WNT-3a Increases iPS-NPC Migration.

After neurosphere harvest and dissociation, DIV 0 mCherry iPS-NPCs were plated on Matrigel® coated transwell inserts (200,000 cells/insert). Cells were allowed to migrate in the presence of SDF-1α (200ng/mL). WNT-3a stimulated mCherry iPS-NPCs migrated significantly more through the Matrigel® coated inserts in the presence of an SDF-1α chemoattractive gradient than control mCherry iPS-NPCs (**Figure 8.8.A, 8.8.B;** n=2/group in triplicate, *student's t-test,* p=0.0137).

Discussion

We hypothesized that FAK overexpression can increase iPS-NPC survival, attachment, migration, and neurite outgrowth. Improvements in these aspects of cellular development and function would be beneficial for iPS-NPC transplant and could improve functional recovery in rodent models of ischemic stroke over current studies.

Because 100% reperfusion does not occur in the ischemic brain within the transplant time window, cells are being transplanted into a region of the brain that is still ischemic to a degree. Ischemic conditions are toxic to transplanted cells and significantly decrease cell survival. Previous studies have demonstrated an important role of FAK signaling that is disrupted in ischemia (Zalewska, Makarewicz et al. 2005, Zalewska, Ziemka-Nałęcz et al. 2005, Hakim, DiMichele et al. 2009), leading to increased cell death. We hypothesized FAK overexpression could increase cell survival in ischemic conditions. We simulated ischemic conditions *in vitro* using oxygen-glucose deprivation. Cell viability was assessed using an MTT assay. We observed no difference in cell viability after the OGD insult between mCherry and mCherry-FAK cells. These data suggest that FAK upregulation in iPS-NPCs does not improve cell survival in ischemic conditions.

Next, we assayed cell attachment. FAK is highly involved in cell attachment and we hypothesized that increased FAK expression in iPS-NPCs would improve cell attachment. Our data in **Figure 8.2** show that FAK overexpression does not improve cell attachment. There is a slight trend that is not statistically significant.

Despite these data, the addition of the FAK inhibitor FAK-14 significantly decreased attachment of both mCherry and mCherry-FAK iPS-NPCs. Taken together, these data suggest that FAK has a necessary role in the attachment of iPS-NPCs, but increased FAK signaling itself is not sufficient to increase attachement in these cells.

We then assayed cellular migration. Many studies highlight the role of FAK and FAK signaling in cellular migration. In a transwell assay, we first observed that migration of both mCherry and mCherry-FAK iPS-NPCs is significantly increased in the presence of an SDF-1α chemoattractive gradient. These data are supported by IHC and western blot data from the previous chapter demonstrating that iPS-NPCs express the SDF-1α complement receptor, CXCR4, at high levels throughout terminal differentiation. These data are exciting because they suggest iPS-NPCs can migrate and home to areas of high SDF-1α content, such as the ischemic core. Second, we observed a trend in increased migration in mCherry-FAK iPS-NPCs compared to mCherry iPS-NPCs. This trend was not statistically significant, suggesting that FAK overexpression is not sufficient to promote increased migration of iPS-NPCs in response to SDF-1α exposure. Third, we observed that FAK-14 addition significantly inhibited migration of iPS-NPCs (both mCherry and mCherry-FAK) in the presence of SDF-1α. FAK-14 inhibition was strong enough that there was no difference between cells that migrated without SDF-1α and those with SDF-1α and FAK-14. These data suggest that FAK plays an essential role in the migration of iPS-NPCs in response to SDF-1α, however overexpression alone is not enough to promote this activity.

As discussed earlier, the involvement of FAK in neurite outgrowth in terms of axonal outgrowth and pathfinding has been previously described (Li, Lee et al. 2004, Ren, Ming et al. 2004, Moore, Zhang et al. 2012). Improved neurite outgrowth and pathfinding is beneficial for transplanted iPS-NPCs. The ischemic brain presents formidable challenges to the integration of iPS-NPCs into host circuitry. Not only is the ECM compromised, but glial scarring also presents a challenge for neurites (axons and dendrites) to extend from transplanted cells and connect with host cells. Mechanisms that improve outgrowth and pathfinding would assist iPS-NPCs in jumping these hurdles. We observe an increase in neurite outgrowth in mCherry-FAK neurospheres 48 hours after plating compared to mCherry neurospheres. Stimulation with Netrin-1 further increases outgrowth in mCherry-FAK neurospheres, however appears to have no effect in mCherry neurospheres. These data suggest that mCherry-FAK iPS cells may exhibit improved neurite outgrowth that warrants additional experimentation.

While we are enthusiastic about iPS transplantation for stroke, many hurdles to successful clinical translation remain. Genetic modifications of iPS cells are one way to address current shortcomings in transplanted cell survival, attachment, and migration. Other strategies mentioned previously include hypoxic preconditioning of cells and incubation with small molecules/peptides/proteins.

Hypoxia preconditioning strategies have been shown to be beneficial in MSC transplantation studies. Hypoxia preconditioning may be beneficial for iPS-NPC transplantation as well. We assay hypoxia preconditioning for iPS-NPCs for the first

time here. We tested 4- and 7-hours of hypoxia preconditioning followed by one hour of reoxygenation. One hour of reoxygenation is crucial for optimization of migratory factor expression. Hypoxia preconditioned iPS-NPCs demonstrate a physiological shift towards increased cell diameter and cell volume compared to normoxic controls. This is intriguing because cell shrinkage is a hallmark symptom of apoptotic cell death. Increases in cell diameter and volume suggest that hypoxia preconditioning may be reducing levels of apoptotic cell death. While these data are exciting, an attachment assay shows there is no increase in healthy adherent cell attachment with hypoxia preconditioning. Data show that in reality the converse happens: there is a trend in decreased attachment with hypoxia preconditioning. We assayed cell death with trypan blue staining in these cells. Despite the Scepter™ observations in cell size, hypoxia preconditioning showed no difference in percentage of trypan blue positive cells, suggesting that hypoxia preconditioning has no effect on cell death. Taken together, these data are puzzling and further experimentation is warranted.

Stimulation of cells with small molecules/peptides/proteins to stimulate adaptive physiological changes before transplantation is another promising strategy to improve transplantation procedures. WNT-3a is highly important in neuronal development, and we hypothesized stimulation of iPS-NPCs with WNT-3a may induce cellular changes that would be beneficial for transplant, such as increased attachment or migration. In a transwell assay with an SDF-1α chemoattractive gradient, cells that were plated in the presence of WNT-3a demonstrated increased migration in response to the gradient compared to control cells. This response is

significant and further experimention into the mechanism and investigations into *in vivo* translation should be undertaken. These data highlight that there are additional approaches to increasing cellular migration before transplantation, which may benefit transplant and functional recovery.

Chapter 9 - Intranasal iPSC Delivery Improves Recovery Following Focal Ischemia

Introduction

iPS-NPC transplantation is an exciting new therapy for ischemic stroke. iPS-NPCs carry the potential for replacement of dead cells in the brain (Chapter 5), augementation of endogenous repair through growth factor secretion (Chapter 6), and the potential for personalized medicine (Dimos, Rodolfa et al. 2008). While current studies show promise, administration route of cells into the brain may not be optimal (Oki, Tatarishvili et al. 2012, Mohamad, Drury-Stewart et al. 2013, Yuan, Liao et al. 2013, Chau, Deveau et al. 2014).

Current transplantation methods focus on intracerebral or intra-vascular transplantation. Intracerebral transplantation is the most direct method to administer cells into the brain. While the method is efficacious, the process of injecting cells into the brain through this route damages healthy brain tissue. Additionally, invasive neurosurgery is needed to access the ischemic stroke region. Surgery opens the skull, meninges, and blood-brain barrier, leaving the patient open to infection and a host of other surgical complications. Intrasvascular transplantation is another commonly used transplantation method. Cells are either delivery through arteries (intra-arterial) or through veins (intra-venous). Disadvantages of this method are that it is inefficient compared to intracerebral transplant, and there is a side effect of cells clumping and depositing in vascular organs such as the lungs, kidneys, and liver. This can cause embolisms and unwanted embolic consequences.

Intranasal delivery (iN or iNA) is a novel non-invasive transplantation route to administer cells into the ischemic brain. Previous studies using MSCs demonstrate that iNA-delivered MSCs can improve functional recovery in animal models of Parkinson's, neonatal hypoxia/ischemia, and ischemic stroke (Danielyan, Schäfer et al. 2009, van Velthoven, Kavelaars et al. 2010, Danielyan, Schäfer et al. 2011, Sun, Wei et al. 2015, Wei, Gu et al. 2015). While the exact mechanism through which cells gain access through the brain is unknown, it is hypothesized that cells migrate along trigeminal and olfactory receptor nerve afferents as well as through perivascular spaces (**Figure 9.1**, below). Our data also suggests that the CXCR4/SDF-1α axis is involved, as hypoxia preconditioning increases CXCR4 expression and specific homing to the ischemic infarct (Wei, Yu et al. 2013)

We demonstrated in Chapter 6 that iPS-NPCs express CXCR4 throughout the stages of differentiation and possess other pro-migratory cellular machinery. We also demonstrated in Chapter 8 that iPS-NPCs migrate towards regions of high [SDF-1α]. SDF-1α is highly expressed in and around the ischemic infarct and provides an endogenous chemoattractive concentration gradient that serves as a migration vector for cells that express CXCR4. Because of these factors we hypothesized that iPS-NPCs can migrate to the ischemic core following intranasal administration and that iNA-iPS delievery can improve functional recovery in an animal model of stroke. Our results are presented in the following chapter.

We also previously discussed the implications of co-morbid affective disorder development and cognitive dysfunction on long-term stroke recovery. In

order to implement successful stroke recovery, it is also necessary to examine and address possible deficits in mood or cognition that may be caused with our stroke model. We used the open-field test one month after stroke induction to assess animals for the development of anxiety-like symptoms with our stroke model. Using the same test, we also evaluate the ability of iNA-iPS cells to alleviate deficits in anxiety-like behaviors induced by ischemic stroke.

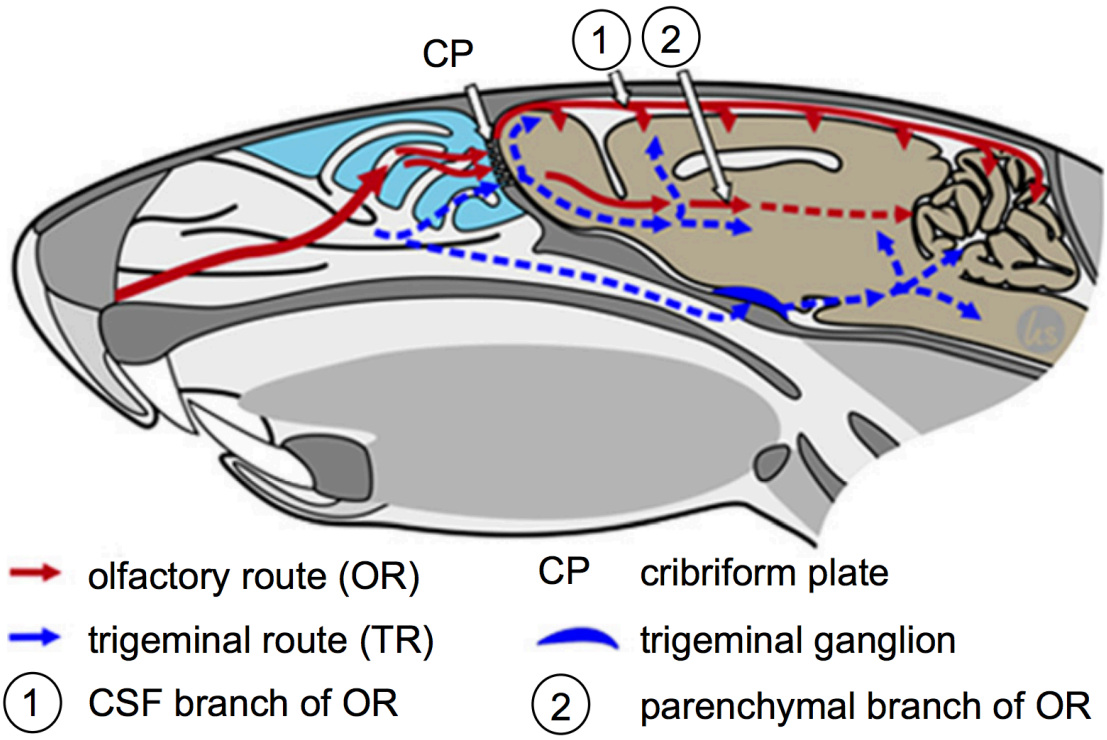


Figure 9.1. Schematic Drawing of Two Routes of iNA Delivery of Cells to the Brain.

After crossing the cribriform plate (CP), the olfactory route (OR, red arrows) divides into two branches: (1) the CSF branch and (2) the parenchymal branch. Solid arrows represent the paths of migration of cells into the brain evidenced in this study, whereas dashed arrows reflect possible hypothetical routes of cell delivery. The hypothetical trigeminal route (TR) consists also of at least two branches one of which crosses the cribriform plate into the parenchyma, where it diverges to the rostral and caudal parts of the brain. The second branch projects from the nasal mucosa to the trigeminal ganglion, where the exogenously applied cells are further distributed to the forebrain, OB and caudal brain areas including the brainstem and

the cerebellum. Reprinted from European Journal of Cell Biology, 88, Danielyan L. et al., Intranasal Delivery of Cells to the Brain, 315-325, 2009, with permission from Elsevier.

Results

iNA iPS-NPCs Reach the Brain and Ischemic Core

iNA delivery of iPS cells into the CNS is currently unreported. Although in previous sections we have demonstrated that iPS-NPCs express pro-migratory machinery and migrate in response to SDF-1α it is unknown if iPS-NPCs can migrate to the ischemic core following iNA administration. iPS-NPCs were pre-labeled with Hoechst 33342 (1:10,000) for 30 minutes before transplantation. Focal ischemic stroke was induced in mice and iPS-NPCs were intranasally delivered 1-4 hours after stroke. 24 hours later, animals were sacrificed, fresh frozen brains sectioned coronally, and sections counterstained with propidium-iodide (PI). We observed Hoechst-positive cells in the perivascular spaces in the dorsal vasculature of the brain (**Figure 9.2.A**). We also observed Hoechst-positive cells in the ischemic infarct (**Figure 9.2.B**). More definite quantitative analysis using mCherry-iPS cells is currently underway but not available at the time of writing.

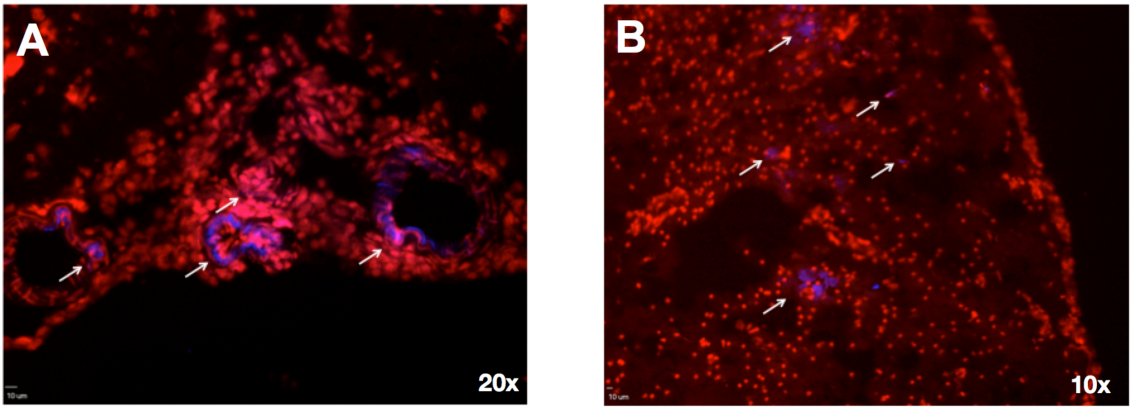


Figure 9.2. iNA iPS-NPCs Reach the Brain and Ischemic Core.

Hoescht-33342 labeled iPS-derived neural progenitor cells enter perivascular spaces (A) and the ischemia core (B) follwing iNA delivery 1-3 hours after ischemic stroke. Images shown are 24 hours post iNA delivery.

iNA-iPS Delivery Increases Local Cerebral Blood Flow (LCBF)

After we confirmed that iNA-iPS-NPCs reached the ischemic infarct (**Figure 9.2.B**), we examined the effect of iNA-iPS delivery on functional recovery. LCBF measurements in infarct region show trends in increased LCBF 3- and 7-days after ischemic stroke and iNA-iPS-delivery (**Figure 9.3**). These trends were not statistically significant (N=2 for 3 days so no statistics were performed; for 7 days, p=0.1361, *students t-test).*

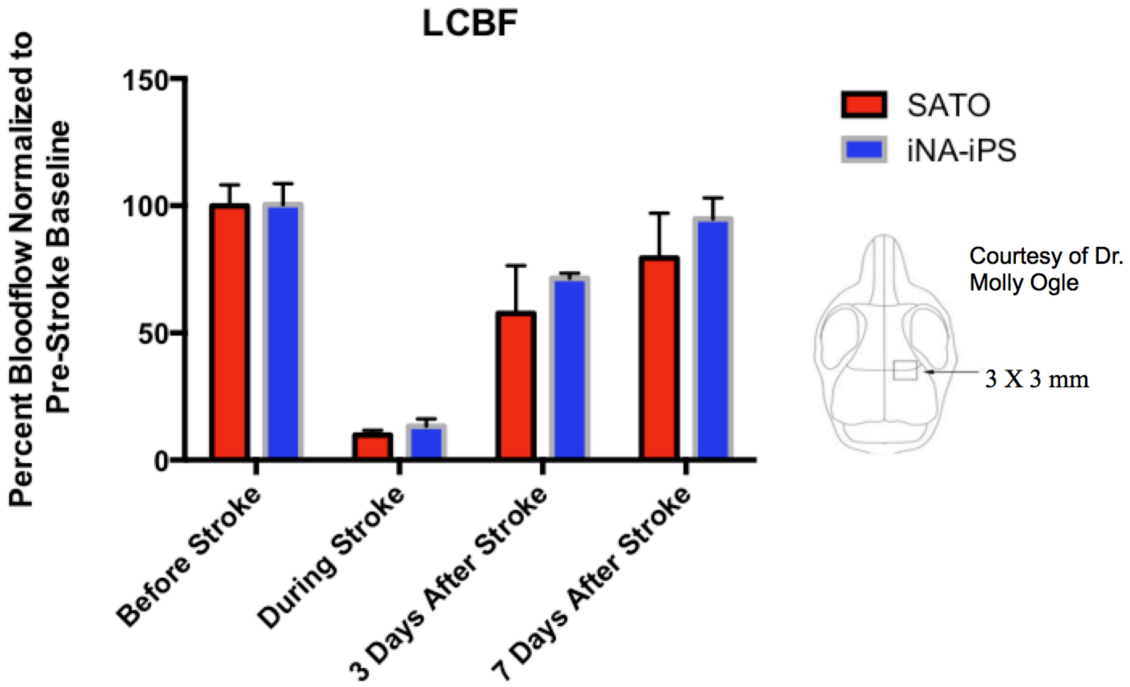


Figure 9.3. iNA-iPS Delivery Increases Local Cerebral Blood Flow (LCBF).

We observed a trend in local cerebral blood flow (LCBF) improvements in the ischemic penumbra at day 3 and day 7 in animals that receive iNA-iPS-NPCs following stroke (n=2-5/group).

iNA-iPS Transplantation Increases Bcl-2 Expression

To follow up LCBF measurements, 72 hours after ischemic stroke and iNA-iPS delivery, animals were sacrificed and tissue from the ischemic penumbra was taken for analysis of pro-survival proteins. We observed a trend in increased Bcl-2 expression (**Figure 9.4.A** and **9.4.B**, however this trend was not significant (n=6/group, *students t-test*, p=0.3143, n.s.). Trends in LCBF and Bcl-2 expression are possibility indicative of increases in functional recovery.

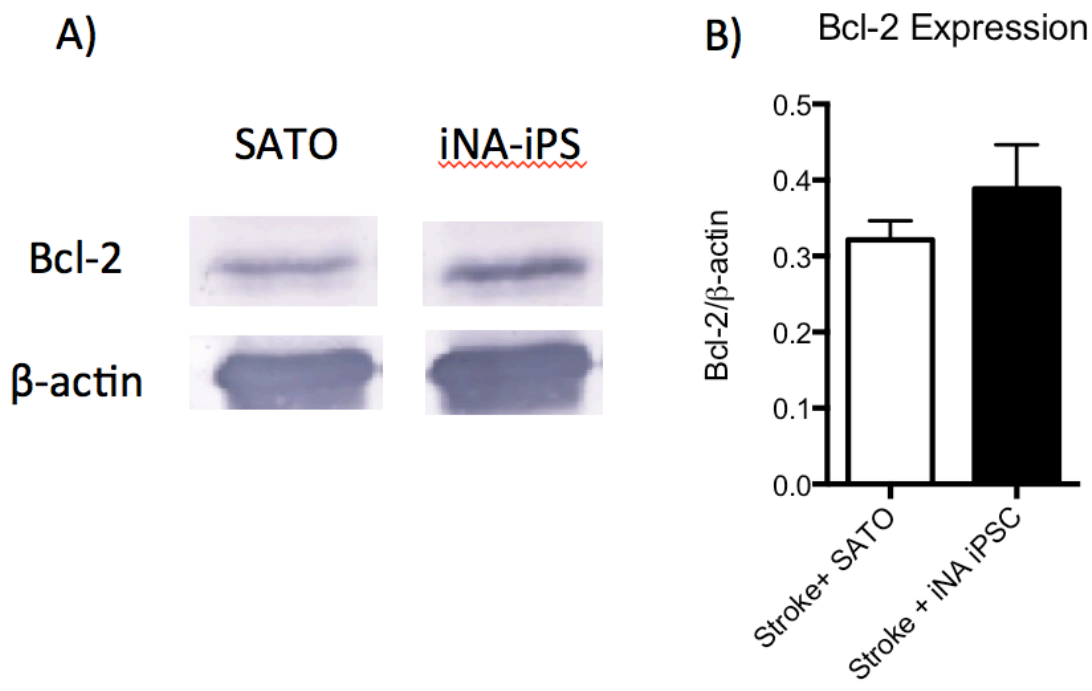


Figure 9.4. iNA-iPA Transplantation Increases Bcl-2 Expression.

72 hours after iNA-iPS delivery, we observed a trend in increased Bcl-2 expression (A, B) in animals that receive iPS therapy compared to animals who receive SATO media only, however this trend fails to reach statistical significance (n=6/group, *students t-test*, p=0.3143).

No Functional Improvement in Animals That Receive iNA-iPS Therapy

To assay functional improvement in ischemic animals, we used the adhesive removal test (Bouet, Boulouard et al. 2009). Because our focal ischemic insults affect the mouse sensorimotor cortex, this test has shown to be sensitive for our stroke model (Mohamad, Drury-Stewart et al. 2013). Despite promising data in the previous figures, no statistically significant differences were observed in contact or removal time in animals that received mCherry iNA-iPS therapy compared to media only at 3-, 7-, 14-, and 21-days after stroke and transplantation (**Figure 9.5.B**). Additionally, animals that received mCherry-FAK-iPS NPCs showed non-significant trends in increased removal time, suggesting mCherry-FAK expression in these cells may not benefit iPS transplantation in our transplantation paradigm as assayed with the adhesive removal test (**Figure 9.5.B**). Although a two-way repeated measure ANOVA is appropriate for this data set, it was not possible to assay every animal at every time point, and missing data points necessitated the use of random one-way ANOVA analysis for each time point separately. One-way random ANOVA showed no statistically significant differences between SATO, mCherry, and mCherry-FAK cells in 3, 7-, 14-, or 21-day contact or 3-, 7-, 14-, 21-day removal times [$p>0.05$, one-way random ANOVA $f(1,6)=2.116$, 2.956, 1.615, 1.864, 3.835, 2.521, 1.845, 2.268 respectively].

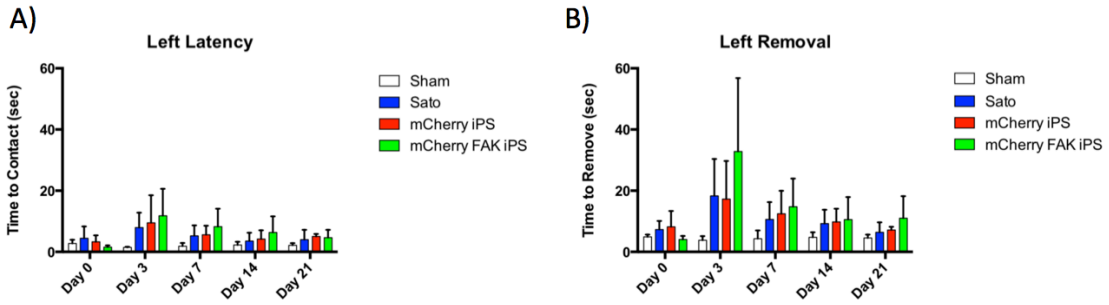


Figure 9.5. No Functional Improvement in Animals That Receive iNA-iPS Therapy.

We assayed functional recovery in animals after stroke with the adhesive removal test. An increase in time to contact (A) or time to remove (B) an adhesive dot from the left forepaw is indicative of the ischemic injury in the sensorimotor cortex. Animals recovered to pre-stroke levels by day 21. There is a small trend in improvement in time to remove in animals that receive iNA-iPS therapy at day 3 after stroke, however this trend is not statistically significant (Sato, mCherry-iPS n=20-23/group; sham, mCherry-FAK n=5; one-way ANOVA $f(1,6)=3.835$, $p>0.05$). iNA mCherry-FAK delivery shows non-significant trends in increased contact and removal times; these data suggest mCherry-FAK expression is not beneficial over iNA mCherry-iPS cells to improve functional recovery following stroke and iNA-iPS delivery.

iNA-iPS Delivery Decreases Endogenous Neurogenesis and Angiogenesis

We have previously demonstrated that iPS-NPC transplant can increase endogenous neurogenesis and angiogenesis in the ischemic penumbra after ischemic stroke. Using IHC, we examined BrdU-positive cells in the ischemic penumbra of animals that received iNA-iPS therapy. 14 days after iNA-iPS therapy, we observe a negative trend in the total number of BrdU-positive cells in the ischemic penumbra (**Figure 9.6.A**), a negative trend in NeuN/BrdU-colabeled cells (**Figure 9.6.B**), and a negative trend in Glut-1/BrdU-colabeled cells (**Figure 9.6.C**). Statistical analysis was not performed on these data as these trends were not statistically meaningful due to lack of power (n=2/group); more experimentation is needed to definitively investigate the effect of iNA-iPS on endogenous neurogenesis and angiogenesis.

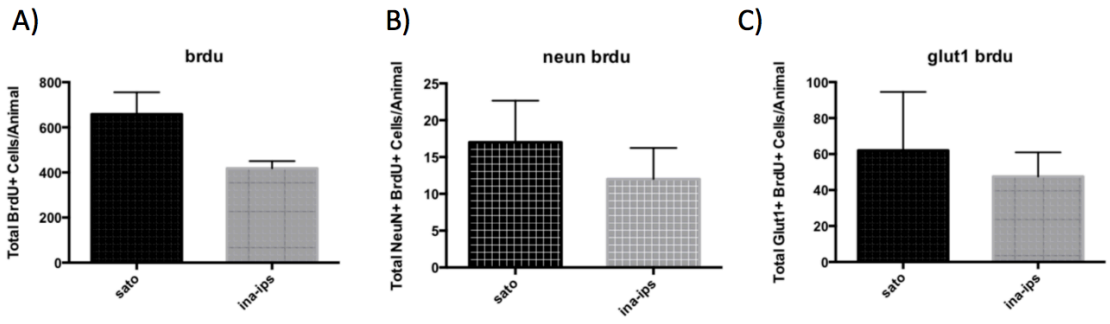


Figure 9.6. iNA-iPS Delivery Decreases BrdU Positive Cells in the Ischemic Penumbra.

We examined the effect of iNA-iPS delivery on endogenous angiogenesis and neurogenesis 14 days after stroke and iNA-iPS delivery. Animals were given daily IP injections of BrdU starting 72 hours after stroke and coronal brain sections stained post-mortem. We observed a trend in preliminary experiments that iNA-iPS delivery decreases the total number of BrdU positive cells (A), the number of NeuN/BrdU co-labeled cells (B) and the number of Glut-1/BrdU positive cells (C, n=2/group for A-C).

No Functional Improvement in Animals That Receive Delayed iNA-iPS Therapy

Because we failed to observe an increase in functional recovery with iNA-iPS delivery 1-4 hours after stroke, we hypothesized that iNA-iPS delivery could be beneficial at a later time point. We examined a delayed delivery time point (72 hours after stroke) to determine the effect of delivery timing on iNA-iPS therapy. iNA-iPS cells (mCherry and mCherry-FAK) were delivered 72 hours after stroke. We observed statistically non-significant trends in increased time to contact and time to remove in animals that received mCherry and mCherry-FAK iNA-iPS cells compared to animals that received SATO media only [n=3-6/group, **Figure 9.7.A and 9.7.B**; two-way repeated measures ANOVA, $f(2, 12)=0.13$, $p>0.05$ for left paw contact and removal]. These data suggest delayed iNA delivery of mCherry or mCherry-FAK iPS-NPCs does not benefit functional recovery as assayed by the adhesive removal test.

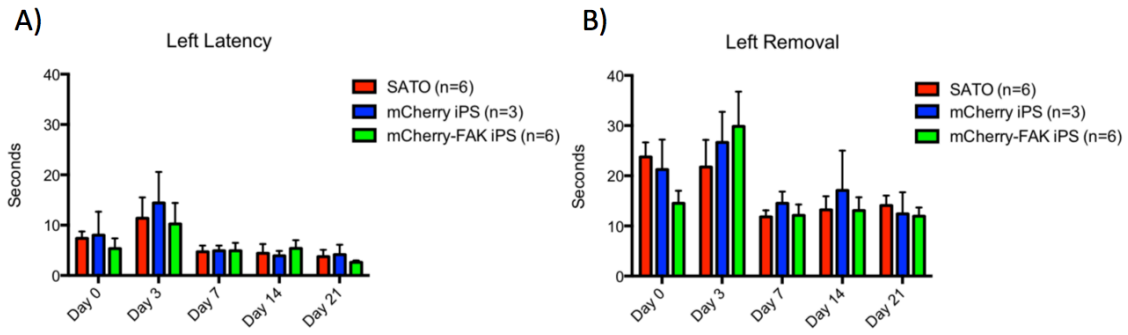


Figure 9.7. No Functional Improvement in Animals That Receive Delayed iNA-iPS Therapy.

We hypothesized delayed iNA-iPS delivery (72 hours after stroke) may have an effect where immediate iNA-iPS delivery did not. Again, we observe no improvement in animals (latency, A, or removal, B) that receive mCherry or mCherry-FAK iNS-iPS delivery [n=3-6/group; two-way repeated measures ANOVA, $f(2, 12)=0.13$, $p>0.05$ for left paw contact and removal].

Delayed iNA-iPS Therapy Has No Effect on BrdU Positive Cells in the Ischemic Penumbra.

Following delayed iNA-iPS therapy, we quantified the total number of BrdU-positive cells in the ischemic penumbra via IHC to determine the effect of delayed iNA-iPS therapy on endogenous neurogenesis and angiogenesis. We observed no difference in the total number of BrdU-positive cells in the ischemic penumbra following delayed iNA-iPS administration (**Figure 9.8.A-C,** n=2/group). Statistical analysis was not performed on these data as these trends were not statistically meaningful due to lack of power (n=2/group); more experimentation is needed to definitively investigate the effect of delayed iNA-iPS delivery on endogenous neurogenesis and angiogenesis.

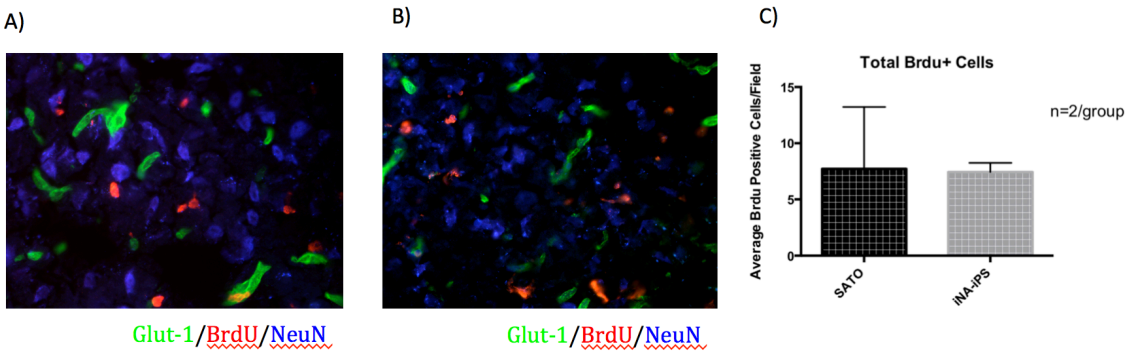


Figure 9.8. Delayed iNA-iPS Therapy Has No Effect on BrdU Positive Cells in the Ischemic Penumbra.

Preliminary data investigating iNA delivery 72 hours after stroke shows no effect on total Brdu levels 14 days after stroke (n=2/group).

iNA-iPS Therapy Reduces Anxiety-Like Behavior

iPS transplantation therapies have potential in exhibiting multiple mechanisms of therapeutic action. Current transplantation studies are focused on neuroprotection and functional recovery, but lack examination on other aspects of stroke such as post-stroke depression. Increased anxiety can be a hallmark of major depressive disorder and affective disorder development following stroke. We used a classical test for anxiety-like behaviors, the open-field test, to assay the effect of iNA-iPS therapy on anxiety-like symptoms following ischemic stroke. We observed that animals (n=5/group) who receive an ischemic insult show deficits in the time they spend on the outside of the box (**Figure 9.9.A**), the time they spend in an inside box (**Figure 9.9.B**), and the number of crosses over from the outside to the inside and vice versa (**Figure 9.9.C**) 21 days after stroke compared to control. iNA-iPS therapy alleviates these deficits and decreases the time animals spend on the outside of the box (**Figure 9.9.A**, n=5/group, one-way ANOVA, F[2,11], p=0.003), increases the amount of time animals spend on the inside (**Figure 9.9.B**, n=5/group, one-way ANOVA, F[2,11], p=0.003), and increases the number of crossing over from the outside to the inside (and vice versa, **Figure 9.9.C**, n=5/group, one-way ANOVA, F[2,11], p=0.0150) compared to stroke media-only animals. No differences in locomotion, i.e. total distance traveled (**Figured 9.10.A**) or average velocity (**Figure 9.10.B**), were observed.

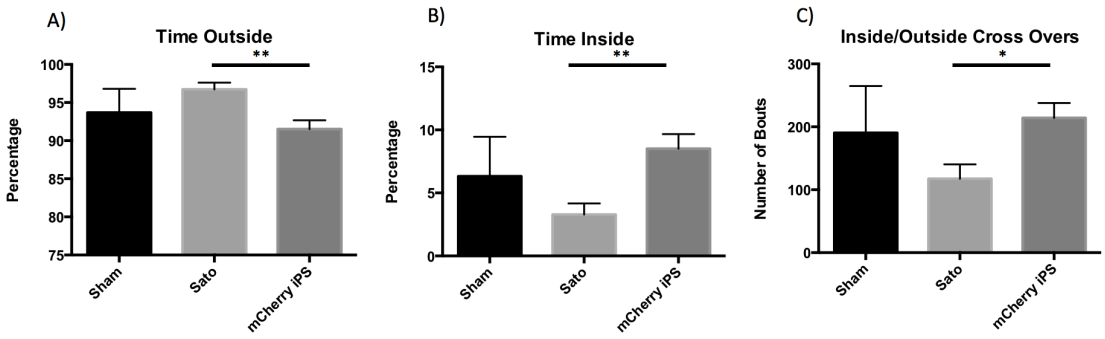


Figure 9.9. iNA-iPS Therapy Reduces Anxiety-Like Behavior.

Animals (4-5/group) were tested for anxiety-like behaviors using an open field test 21 days after stroke and transplantation. Animals that received mCherry-iPS cells spent more time on the outside (A), more time in the center of the field (B), and crossed over from the inside to outside (C) more than animals who received vehicle alone (one-way ANOVA, F[2,11], p=0.003, 0.003, and 0.0150 respectively). These behaviors are indicative of our model causing anxiety-like behaviors in animals that can be alleviated by iNA-iPS delivery after stroke.

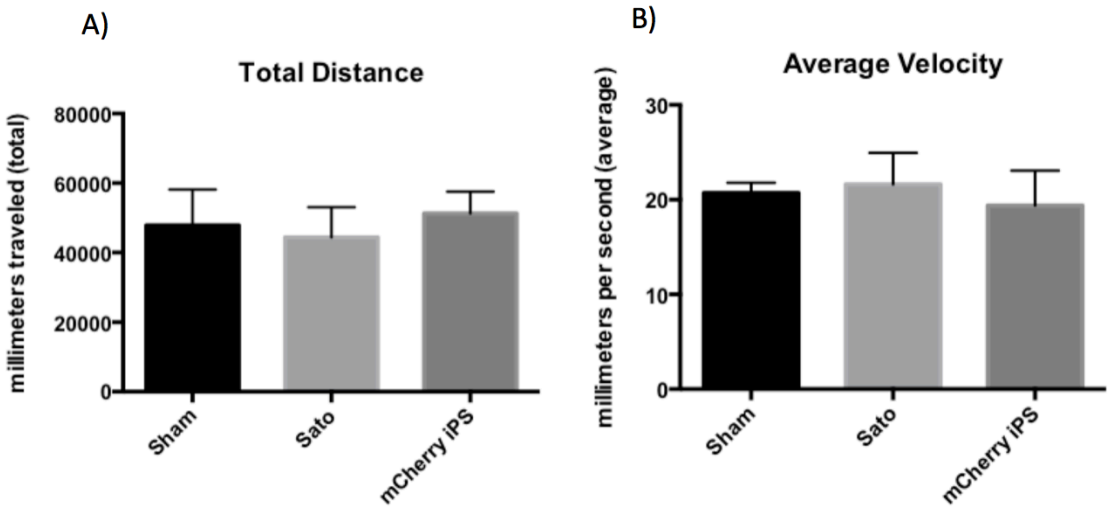


Figure 9.10. No Differences In Locomotion in Open Field Test.

No differences in locomotor behavior (total distance, A, and average velocity, B) in the open field test were observed (n=4-5/group), suggesting the previous results were related to anxiety-like behaviors and not global deficits in locomotion.

Delayed Intranasal Transplant Improves Anxiety Like Behaviors

We also observed the effect of delayed iNA-iPS therapy (72 hours after stroke) on anxiety-like behaviors. We observed similar results to those shown in **Figure 9.9** for the mCherry iPS groups compared to SATO and sham. Trends were observed in decreased distance traveled on the outside of the box compared to media-only controls [n=3-6/group, **Figure 9.11.A**; one-way random ANOVA $f(3,16) = 0.2176$, $p>0.05$]; increased distance on the inside of the box compared to media-only controls [n=3-6/group, **Figure 9.11.B**; one-way random ANOVA $f(3,16) = 3.226$, $p>0.05$]; decreased velocity in the inside of the box compared to media-only controls [n=3-6/group, **Figure 9.11.D**; one-way random ANOVA $f(3,16) = 0.6682$, $p>0.05$]; and finally crosses over from the outside to the inside and vice versa compared to media-only controls [n=3-6/group, **Figure 9.11.F**; one-way random ANOVA $f(3,16) = 3.730$, $p>0.05$]. These trends were not statistically significant. Animals who received iNA mCherry-FAK cells showed decreased velocity on the outside of the box compared to sham [n=6, one-way random ANOVA $f(3,16) = 4.766$, $p=0.0133$], decreased turns compared to sham and SATO [n=6, one-way random ANOVA $f(3,16) = 5.907$, $p=0.0076$, 0.0177 respectively], and decreased crossing over from the outside to the inside compared to sham [n=6, one-way random ANOVA $f(3,16) = 3.370$, $p=0.0382$]. These data suggest that the animals that received iNA mCherry-FAK iPS cells may have some sort of locomotor impairment that interferes with their performance in this test. Further experimentation is necessary to elucidate the underlying cause.

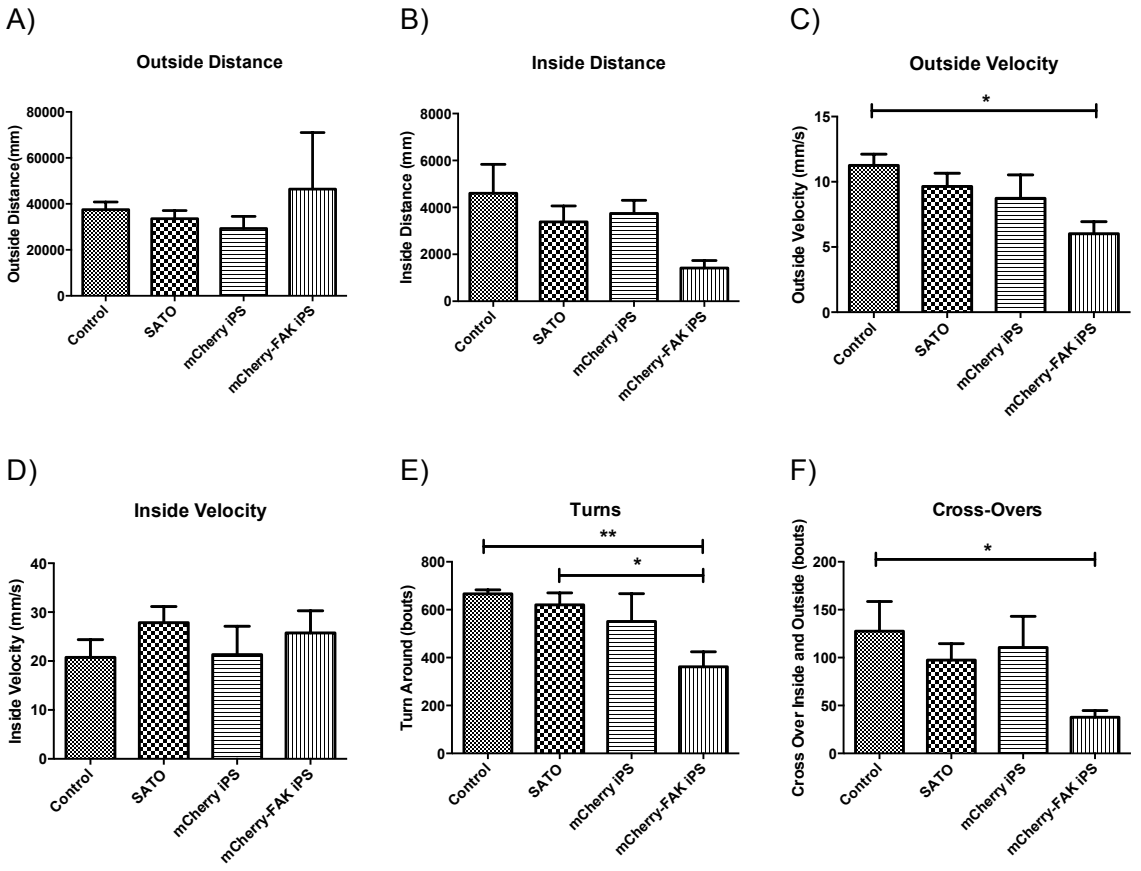


Figure 9.11. Delayed Intranasal Transplantation Improves Anxiety-Like Behaviors After Stroke.

We observed trends in increases in inside distance (B), inside velocity (D), and crosses from outside to inside (F) in animals that receive mCherry delayed iNA-iPS therapy compared to SATO control (n=3-6/group). This behavior is indicative in decreases in anxiety-like behavior. Animals that received delayed iNA mCherry-FAK-iPS showed significantly decreased velocity around the outside compared to sham (p=0.0133), decreased turns compared to sham and SATO groups (p=0.0076, 0.0177 respectively), and decreased cross-overs compared to sham (p=0.0382).

Discussion

We demonstrate here for the first time that iNA-iPS-NPCs migrate into the CNS following ischemic stroke as a proof-of-principal (**Figure 9.2**). Cells labeled with Hoechst-33342 were delivered 1-4 hours after stroke were observed in the perivascular spaces as well as the ischemic core itself. We were excited about these results and moved forward with examinations on functional recovery. Further experimentation is underway to produce a quantitative sense of transplantation, however was not available at the time of writing.

We observed trends in increased LCBF 3- and 7-days after ischemic stroke and transplantation (**Figure 9.3**). Although not statistically significant, these trends indicate that iNA-iPS therapy may improve functional outcomes at later time points. The mechanism by which iNA-iPS therapy would increase LCBF at these time points is unclear, however these data can most likely be attributed to a neuroprotective effect of cell transplantation. We examined pro-survival/anti-apoptotic protein expression in the ischemic penumbra to confirm our hypothesis. Western blot data demonstrates that the pro-survival/anti-apoptotic gene Bcl-2 is upregulated in the ischemic penumbra in animals who receive iNA-iPS therapy compared to media-only 72 hours after stroke/transplantation (**Figure 9.4**), however these data were not statistically significant.

Functional recovery was assayed with the adhesive-removal test (Bouet, Boulouard et al. 2009). Our stroke model affects the rodent somatosensory cortex (predominantly the whiskey barrel cortex) and this test has been seen to be sensitive to our stroke model (Mohamad, Drury-Stewart et al. 2013). We observed

a slight decrease in left-paw removal time (indicative of improvement in right-hemisphere stroke) but this was not significant. No differences were observed at later time points (**Figure 9.5**). These data suggest that mCherry or mCherry-FAK iNA-iPS therapy 1-4 hours after stroke does not improve functional recovery as assayed by the adhesive removal test. It is possible other behavioral tests may show a difference. We also tried the corner test, however animals showed a bias towards turning before stroke and we were unable to use this test. The cylinder test was also tested, however animals again showed a "handedness" and we were unable to use this test.

We have previously shown that iPS transplantation can increase endogenous angiogenesis and neurogenesis after stroke (Chau, Deveau et al. 2014). In this study, we gave a small subset of animals daily BrdU injections starting 72 hours after stroke to label new-born endogenous cells. 14 days after stroke, we stained coronal brain sections in the animals (**Figure 9.6**). We observed a decreased trend in total BrdU-positive cells, BrdU/NeuN co-labeled cells, and BrdU-Glut-1 co-labeled cells. Although the sample size is small (n=2), these data suggest that iNA-iPS therapy 1-4 hours after stroke is not beneficial to endogenous neurogenesis and angiogenesis.

1-4 hour delivery of iNA-iPS cells may be too soon after the stroke injury and has questionable clinical relevance. We also examined delayed iNA-iPS transplantation to test if delivery timing had any effect. With the adhesive removal test, we again observed no differences between control animals and animals that received mCherry or mCherry-FAK iNA-iPS therapy (**Figure 9.7**). We examined

endogenous angiogenesis and neurogenesis 14 days after stroke (11 days after iNA-iPS therapy) and observed no differences in total BrdU-positive cells in the ischemic penumbra (**Figure 9.8**).

Stroke is a complex disorder that has implications beyond neural circuit repair and the direct functional recovery of damaged circuits. As many as a third of stroke survivors suffer post-stroke depression (Roger, Go et al. 2011, Go, Mozaffarian et al. 2013); post-stroke depression and other affective disorders (bipolar disorder, generalized anxiety disorder) can be debilitating to recovery efforts and increase the burden of palliative care. Increased anxiety can be a major symptom of major depression. We examined anxiety-like symptoms in the animals and turned our attention to alleviation of these symptoms because iNA-iPS therapy had no effect on functional recovery.

The open-field test is a commonly employed assay of anxiety-like behaviors in rodents. Animals in our studies were assayed with the open field test (**Figure 9.9, 9.10,** and **9.11**) 21 days after stroke. We examined in all cases that our focal ischemic stroke model induced symptoms of anxiety-like behaviors in injured animals. Animals who received a focal ischemic insult spent more time on the outside of an open field, less time on the inside, and crossed over from the inside to the outside less than control animals. These behaviors are indicative of anxiety-like behaviors. Immediate iNA-iPS delivery after stroke (1-4 hours) alleviates these symptoms by increasing the amount of time the animals spent on the inside of the box, decreasing the amount of time the animals spent on the outside of the box, and increasing the crossing-overs between the outside and the inside. We observed no

differences in mean distance traveled or average velocity, suggesting that the results we observed were not due to mean changes in animal locomotion. Similar trends were observed in animals that received iNA-iPS therapy 72 hours after stroke, however these results were only trends and were not statistically significant. mCherry-FAK-iPS delivery appeared to decrease locomotive behaviors in the test, causing animals to perform worse in the tasks. Further experimentation is necessary to examine the effect of iNA mCherry-FAK delivery on locomotion. Taken together, these data support an additional mechanism of iPS therapy, the alleviation of long-term anxiety-like symptoms. The mechanisms by which this occurs is currently unknown, however warrants further investigation.

In summary, iNA-iPS therapy is a promising novel therapeutic avenue for ischemic stroke. Preliminary data show potential but fall short on delivering observable differences in functional recovery. This suggests that additional functional recovery assays should be employed or that therapy should be optimized further. We attempted to optimize iNA-iPS delivery through genetic overexpression of FAK, however animals that received these cells faired no better in all of the behavioral measures examined. Additional optimizations, or perhaps combinatorial therapy approaches that combine transplantation with additional therapy, such as peripheral whisker stimulation, may be beneficial. We have previously demonstrated the effects of peripheral stimulation in functional circuit repair (Whitaker, Cui et al. 2006). The combination of whisker stimulation with iPS transplantation may improve functional recovery over iPS transplant alone (especially intranasal transplant), and studies are currently underway exploring this

possibility. Although no differences were seen in functional recovery, we did observe significant alleviation of anxiety-like symptoms following iNA-iPS delivery. These data highlight an additional mechanism that iPS-therapy acts on, and warrants further study.

Chapter 10 - Summary and Conclusions

The body of work presented in this dissertation accomplishes several objectives. First, we further characterize the modified MCAo stroke model used in our lab and examine the role of CCA occlusion in infarct development. Second, we show that iPS-NPCs can efficiently be differentiated into functional neurons. These cells express mature neuronal markers and express other pro-migratory proteins. We also characterize FAK expression in these cells, a protein that's highly important in cell survival, migration, adhesion, and neurite outgrowth. Third, we demonstrate the efficient generation of stable mouse iPS cells with episomal vectors and transfection. This is currently not reported in the literature for mouse iPS cells. Fourth, we demonstrate that iPS-NPCs migrate towards high [SDF-1α] and that migration can be blocked with the specific FAK inhibitor FAK-14. Cell adhesion is also blocked with FAK inhibition via FAK-14. Although FAK overexpression does not increase survival, adhesion, or migration, FAK-14 data suggests that FAK is still an essential part of these physiological processes. Fifth, we demonstrate an observation that neurite outgrowth is increased with FAK overexpression. This may be beneficial for iPS-NPC graft integration but further investigation is needed. Genetic upregulation is one way to optimize cells for transplant, and we examine other methods such as hypoxia preconditioning and WNT-3a stimultation. Hypoxia preconditioning shows physiologic data suggesting decreased cell death, but attachment and trypan blue assays are contradictory. These experiments are puzzling and warrant further investigation.

In vivo, we make the important observation that iPS cells reach the CNS and ischemic infarct following iNA delivery. iNS-iPS therapy appears to improve LCBF

and pro-survival protein upregulation 3- and 7-days after stroke/transplantation, but these observations warrant further investigation. Despite these observations, iPS-NPC therapy does not appear to improve functional recovery assayed by the adhesive removal test. Additional testing is warranted. We also examined delayed (72 hour) iNA-iPA therapy. Delayed iNA-iPS therapy also showed no improvements in functional recovery through the adhesive removal test. It would be worth exploring additional optimization of iNA-iPS therapy, including (but not limited to) dosage variations, timing variations, and small molecule/peptide/protein stimulation (such as WNT-3a).

We hypothesized that FAK overexpression would improve iPS therapy and increase functional recovery after transplant in a rodent model of focal ischemia. Interestingly, animals that received mCherry-FAK-iPS cells faired worse in all of the behavioral assessments. Additional experimentation is needed to determine the cause of these observations. *In vitro* data suggest that although FAK overexpression is not beneficial, that FAK is necessary for these processes. As such, we wouldn't expect FAK overexpression to worse functional deficits by itself.

Although we observed no functional improvements through the adhesive removal test, we did observe that iNA-iPS alleviated anxiety-like behaviors in animals as assayed with the open-field test. Additional experimentation is needed to elucidate the mechanism by which this may be occurring. This is an important (and currently unreported/unexplored) observation highlighting the multiple mechanisms of action possessed by iPS therapies that would be worth exploring in

future studies. Other combinatorial approaches, such as the combination of iPS transplant with peripheral whisker stimulation are currently unreported. Studies are currently underway.

This dissertation has discussed and alluded to stem cell therapy possessing multiple mechanisms of action. Studies involving stem cell therapies for stroke have previously focused on functional recovery, and lack mechanistic insight beyond observations of cell survival and assays reporting behavioral outcomes. The remaining segments of this dissertation are dedicated to putative mechanisms of action of stem cell therapy, especially iPSC transplantation.

The following is adapted with permission from:

Deveau TC, Yu SP, Wei L. "Cellular Therapy for Ischemic Stroke" Translational Stroke Research: From Target Selection to Clinical Trials (Springer Series in Translational Stroke Research) by Paul A. Lapchak and John H. Zhang. Springer Publishing March 23, 2012. pp. 777-817. DOI : 10.1007/978-1-4419-9530-8.

Putative Underlying Mechanisms for iPS-Based and Other Cellular Therapies

The original goal of cellular therapy for stroke was cellular replacement. Researchers aimed to utilize stem cells to replace lost tissue and regenerate lost neuronal circuits. Integration of transplanted cells has been demonstrated, and future studies should examine the efficiency of integration and the terminal phenotype of transplanted cells. As the field matures, focus has shifted away from cellular replacement and is moving toward the use of stem cells for cellular augmentation. Research has demonstrated that cellular therapy can be efficacious

without replacing lost circuits. One study even documented functional improvement in rats following ischemia despite the fact that the murine NSC grafts were undectable at 6 months, suggesting that recovery was mediated by mechanisms aside from circuit integration (Ramos-Cabrer, Justicia et al. 2010). Stem cells hold great promise for novel stroke therapeutics because of the extended window of time for efficacy, multiples mechanisms of action, and the ability to adapt to their environment. It is now clear that exogenous and endogenous cellular delivery can improve prognosis in animal models as a result of trophic support, angiogenic stimulation, increases in endogenous neurogenesis, and inflammatory modulation.

Cell Replacement

We have demonstrated that iPS cells can be efficiently differentiated into functional neurons *in vitro* that survive and differentiate into neurons *in vivo*. It has been shown that transplanted cells integrate into the host circuitry as well (Yuan, Liao et al. 2013). These are important and expected observations. There are many important questions remaining. What is the percentage of transplanted cells that successfully integrate? What is the percentage of cells that survival transplant? What is the terminal phenotype of the cells? These are important questions that need to be addressed by future experiments.

Trophic Support

Trophic factors such as vascular endothelial growth factory (VEGF), nerve growth factor (NGF), and brain-derived neurotrphic factor (BDNF) are important for the growth of vascular and neuronal tissue in the CNS. We reported in Chapter 6 that iPS-NPCs and neurons express BDNF and VEGF. VEGF is important for vascular growth and angiogenesis (Wei, Erinjeri et al. 2001). Under non-pathological conditions, neurotrophins such as NGF and BDNF "promote survival, differentiation, and neurite extension in many types of mammalian central nervous system neurons" (Schäbitz, Schwab et al. 1997). Following ischemia, VEGF and BDNF are up-regulated among others (Zhang and Chopp 2002, Kaya, Gürsoy-Ozdemir et al. 2005). Exogenous administration of growth factors themselves has been demonstrated to be beneficial in animal models of ischemia (Schäbitz, Schwab et al. 1997, Schäbitz, Sommer et al. 2000, Kaya, Gürsoy-Ozdemir et al. 2005). Furthermore, studies involving cellular therapy (MSCs especially) have demonstrated that transplanted cells exert beneficial effects, at least in part, through increased secretion of trophic factors (Wakabayashi, Nagai et al. 2010). Increases in trophic factor production through cellular therapy may prove beneficial for cerebral ischemia (Caplan and Dennis 2006).

For example, increases in trophic factor production are seen in several studies following MSC transplantation post-ischemia. Increases in VEGF expression are observed in peri-infarct tissue 14 days after ischemia (pMCAO) in rats treated

with IV or intracarotid BM-MSCs compared with non-treated (Gutiérrez-Fernández, Rodríguez-Frutos et al. 2010). A study by Wakabayashi and colleagues (2010) demonstrated that animals receiving IV hMSC transplants following transient MCAO had smaller infarcts, improved recovery, and increased expression of VEGF, EGF, and bEGF compared with controls (Wakabayashi, Nagai et al. 2010).

Evidence to date suggests that increasing trophic factor expression through cellular transplantation may exert therapeutic efficacy in animal models of ischemia. However, trophic factor actions can also be detrimental to prognosis, depending on the timing. VEGF, for example, can increase BBB permeability and the risk of hemorrhagic transformation (Kaya, Gürsoy-Ozdemir et al. 2005). Therefore, future studies are needed on specific windows of time across cell types that trophic factor induction is beneficial.

Angiogenesis

Following stroke in rodent models, there is an innate increase in angiogenesis in the ischemic border. Surface collateral growth and parenchymal reperfusion increases are observed, indicative of post-stroke angiogenesis (Wei, Erinjeri et al. 2001). Brdu/endothelial co-labeling also increases, peaking at 7 days in the rat (Wei, Erinjeri et al. 2001). Increases in angiogenesis are also observed in human patients following ischemia (Krupinski, Kaluza et al. 1994). Boosting the innate angiogenic response following ischemia would ostensibly be beneficial for

ischemic stroke therapy by increasing blood flow, reducing infarct volume, and supporting neural vascular networks for quicker neurological recovery (Navaratna, Guo et al. 2009). Additionally, increases in angiogenesis in non-infarcted tissue may be beneficial after ischemia as there is a well-known correlation with post-ischemic dementia and reduced blood flow in non-infarcted areas of the brain (Schmidt, Schmidt et al. 2000, Navaratna, Guo et al. 2009).

Several studies have shown the effect of stem cell transplantion and increased angiogenesis in several animal models of disease, especially with MSC transplantation. A 2007 study showed that MSC transplantation in an excisional wound splinting model in mice increased capillary density after transplantation (Wu, Chen et al. 2007). Transplantation of allogenic fetal-membrane-derived and bone-marrow-derived MSCs in a rat model of hindlimb ischemia increased capillary density three weeks after injection (Ishikane, Ohnishi et al. 2008). Hu et al demonstrated that cardiac transplantation of murine BM-MSCs in a rodent model of myocardial infaction increased vessel density/area fraction in the heart 6 weeks after transplantation (Hu, Yu et al. 2008). Suzuki et al showed increases in hind limb blood flow in a model of hind limb ischemia after transplantation of iPS cell-derived Flk-1 positive cells (Suzuki, Shibata et al. 2010). Increases in angiogenesis and capillary density have also been observed in rat MCAO models following intravenous allogenic BM-MSC transplantion (Komatsu, Honmou et al. 2010).

Literature suggests a relationship between angiogensis and prognosis in animal models and in human patients, highlighting the putative importance of

angiogenesis and post-ischemic recovery. Evidence to date suggests that stem cell transplantation can cause increases in angiogenesis. We previously observed iPSC transplantation can increase endogenous angiogenesis (Chau, Deveau et al. 2014). Surprisingly in the present study, increases in endogenous angiogenesis were not observed. Future studies are needed to fully elucidate the mechanisms behind stem cell-mediated increases in angiogenesis and any possible relationship between stem cell-mediated angiogenesis and functional recovery.

Modulation of Endogenous Neurogenesis

As mentioned previously, the adult CNS is capable of generating new neurons and glia from progenitor cells in several regions. Of particular interest in cerebral ischemia are neural progenitors from the sub-ventricular zone (SVZ). Under non-pathological conditions, neural progenitors from the SVZ migrate long distances along the rostral migratory stream (RMS) where they reach the olfactory bulb. In the olfactory bulb, SVZ-derived NPCs that survive differentiate into inhibitory granule and periglomular interneurons (Lledo, Alonso et al. 2006).

It is now known that endogenous neurogenesis in the adult SVZ is regulated by stroke itself [for a review see (Wiltrout, Lang et al. 2007, Zhang, Zhang et al. 2008)]. Ischemia not only increases innate neurogenesis in the SVZ, but also diverts migrating NPCs away from the RMS towards the infarct. Unfortunately, the amount of new cells that survive is small and only a minute amount of dead neurons are

replaced (Haas, Weidner et al. 2005). One study by Arvidsson and colleagues demonstrated in a rat MCAO model of ischemia that only about 60% of migrating neural progenitors survived in the striatum 6 weeks after ischemia, replacing an estimated 0.2% of the dead neurons (Arvidsson, Collin et al. 2002). Ischemia has also been known to regulate neurogenesis in the SGZ and SCZ. Upregulation of neurogenesis in the SGZ has been observed following cerebral ischemia in different animals and in different animal models of ischemia (Liu et al 1998, Jin et al 2001, Li et al 2010). Nakatomi and colleagues demonstrated the contribution of cells from the pPV (part of the SCZ) in repopulating hippocampal cells following ischemia (Nakatomi, Kuriu et al. 2002).

Several cytokine and chemotaxic gradients are known to regulate NPC upregulation and infarct migration following ischemia. The CXCR4/SDF-1α (CXCL12) axis is one chemotaxic gradient involved in migration of neural progenitors to the infarct. Following ischemia, stromal cell derived factor 1-α is upregulated, attracting neural precursor cells expressing the complement receptor CXCR4 (Imitola, Raddassi et al. 2004, Robin, Zhang et al. 2006). Increasing the strength and/or duration of chemotactic gradients may be one method to improve the efficacy of endogenous NPCs following ischemia.

A number of studies have demonstrated that endogenous neurogenesis and migration can be upregulated following ischemia by a number of mechanisms. For example, use of cytokines (Gonzalez-Perez, Quiñones-Hinojosa et al. 2010), growth factors (Schäbitz, Steigleder et al. 2007), small molecules (Tanaka, Tanaka et al.

2010), environmental enrichment (Hicks, Hewlett et al. 2007), and peripheral stimulation (Li, Yu et al. 2008) has all demonstrated increases in endogenous neurogenesis following ischemia. Transplantation of exogenous cells has also been known to increase innate neurogenesis (Jin, Xie et al. 2010).

The exact contribution of SGZ neurogenesis to ischemic stroke recovery is unclear. However, Li and colleagues recently demonstrated that reducing SVZ and SGZ neurogenesis by ICV Ara-C infusion (a cell proliferation inhibitor) increased the size of the infarct and worsened neurological deficits following ischemia (Li, Piao et al. 2010). It is reasonable to suggest that post-ischemia neurogenesis would be beneficial for prognosis.

We previously observed iPSC transplantation can increase endogenous neurogenesis (Chau, Deveau et al. 2014). Surprisingly in the present study, increases in endogenous neurogenesis were not observed. Future studies should examine methods to increase the innate neurogenic response, increase the migration of NPCs, and increase the survival of NPCs.

Immune Modulation

The effects of pro- and anti-inflammatory cytokines were discussed in the previous sections. Therapies for stroke that can reduce pro-inflammatory cytokines and/or increase anti-inflammatory cytokines at certain times following ischemia could be beneficial for prognosis. Several studies have demonstrated the effect of

exogenous cell transplantation and modulation of neuroinflammation following ischemia in different cell types. iPS transplantation with fibrin glue can reduce pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-2) and increase anti-inflammatory cytokine production (IL-10, iNOS, IL-4) 1 week after transplantation in an MCAO model in 8-week old rats (Chen, Chang et al. 2010). Non-human primate ischemia models have also seen immune influence by transplanted stem cells. Li et al demonstrated in *Macaca Fascicularis* males that intracerebral human BM-MSC transplantation following ischemia decreases GFAP reactivity and increases IL-10 positive cells and mRNA levels (Li, Zhu et al. 2010). Examination of immune modulation after iPS transplant is currently under reported and further investigation is warranted. There is a link between depression and immune system dis-regulation in humans, and immune modulation by iPSCs could be an underlying mechanism of the observed alleviations in anxiety-like behaviors observed in the present study.

Chapter 11 - References

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