Transplantation of Directly Reprogrammed Human Neural Precursor Cells to Promote Repair in a Preclinical Model of Stroke

by

Ilan Vonderwalde

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract:

Stroke is one of the leading causes of acquired long-term disability worldwide. Cell transplantation is a promising therapeutic intervention. Herein, we explore the efficacy of a novel population of directly reprogrammed human neural precursor cells (drNPCs) to treat the stroke-injured brain. First, we confirmed that drNPCs are neurally committed and established a working model of focal ischemia in SCID/Beige mice that leads to sustained functional deficits. We then transplanted cells 4 days after stroke to test for therapeutic benefits. Sensorimotor assessment, immunostaining, and lesion volume outcomes were used to measure functional recovery, cell survival and differentiation, and tissue repair. Our results demonstrate that drNPCs survive up to one month within the transplanted tissue, primarily differentiate into astrocytes in vivo, promote functional recovery, and do not affect the lesion volume following stroke. These results indicate that drNPCs may be a viable source of cells for clinical application.
Contributions

This project would not have been possible without the contributions of my peers. Credit is due to those that helped this study come to where it is today.

Ashkan Azimi did the qPCR and RT-PCR to characterize sister cultures of transplanted cells *in vitro* and optimized some of the immunostaining procedures. Gabrielle Rolvink aided with sectioning, imaging, and staining. In addition, she conducted the measurement of gliosis and injury volumes to perform the lesion volume analysis. Monoleena Khan and Ritika Kompella also helped with tissue sectioning and immunostaining. Ricky Siu assisted with surgeries. Priya Anandakumaran, Ana Fokina, and Tobias Fuehrmann helped with HAMC preparation.
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Lastly, thank you to my beard for keeping me warm in the winter and hip in the summer. You were always a great topic of conversation but I think it’s time for you to go.
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<th>Description</th>
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<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>BMSC</td>
<td>bone marrow stem cell</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>drNPC</td>
<td>directly reprogrammed neural precursor cell</td>
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<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HAMC</td>
<td>hyaluronan methylcellulose</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>hUCBC</td>
<td>human umbilical cord blood cell</td>
</tr>
<tr>
<td>HuNu</td>
<td>human nuclei</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NPC</td>
<td>neural precursor cell</td>
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<tr>
<td>NSC</td>
<td>neural stem cell</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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PCR          polymerase chain reaction
PVD          pial vessel disruption
ROUT         robust regression and outlier removal
RT-PCR       reverse transcription polymerase chain reaction
RT-qPCR      quantitative reverse transcription polymerase chain reaction
tPA          tissue plasminogen activator
SCID         severe combined immunodeficiency
STDEV        standard deviation
STAIR        stroke therapy academic industry roundtable
STEPS        stem cell therapies as an emerging paradigm in stroke
SVZ          subventricular zone
1. Introduction

A stroke occurs when blood flow to the brain is interrupted. This can happen through a hemorrhagic stroke, which occurs when a blood vessel in the brain ruptures, or through an ischemic stroke, which occurs when blood flow to the brain is occluded. Hemorrhagic strokes lead to increased pressure on the brain due to leaked blood and are associated with increased risk of mortality (Andersen et al., 2009; Kiyohara et al., 2003). Ischemic strokes, on the other hand, lead to functional disabilities due to decreased glucose and oxygen delivery to the brain, resulting in rapid cell death, and ultimately, impaired neural function. Current treatment strategies offer limited success, making stroke one of the leading causes of acquired long-term disability worldwide (Mackay and Mensah, 2004; Mendis, 2013; Mozaffarian et al., 2015).

Ischemic strokes are the most prevalent type of stroke, accounting for roughly 87% of total cases in human patients (Mozaffarian et al., 2015). Due to the nature of rapid cell death, if untreated, ischemic stroke results in devastating long term physical deficits (Min and Min, 2015), cognitive impairments (Weinstein et al., 2014), and psychological disturbances (Cumming et al., 2015; Hackett and Pickles, 2014; Hackett et al., 2005; Vataja et al., 2004). Unfortunately, there is no cure for the long-term disabilities that result following stroke (Mendis, 2013; Mozaffarian et al., 2015). In 1998, it was reported that approximately 50,000 strokes occur in Canada annually (Hakim et al., 1998), and in 2009, roughly 300,000 Canadians reported suffering from the disabling effects of a stroke (Public Health Agency of Canada, 2009, 2011). The lost productivity and caregiver costs account for 28% of stroke’s economic burden (Mittmann et al., 2012) in addition to the significant burden on the healthcare system and impact of stroke on quality of life (Almkvist Muren et al., 2008; Public Health Agency of Canada, 2009, 2011; Carod-Artal and Egido, 2009; Cerniauskaite et al., 2012; Haley et al., 2011; Min and Min, 2015). Thus, it is important to identify strategies that will reduce the long-term disabilities that result from stroke.

Cell-based interventions to repair the injured brain and promote recovery have therapeutic potential and the capacity to change the way stroke is treated in the clinic (Dailey et al., 2013a; Dibajnia and Morshead, 2012; Janowski et al., 2015; Kenmuir and Wechsler, 2017; Lindvall and Kokaia, 2011; Meamar et al., 2013; Rosado-de-Castro et al., 2013; Savitz et al., 2004). One potential cell-based approach involves transplantation of cells to the stroke injured central nervous system (CNS). Limitations to this strategy do exist, with one of the most
significant challenges being the identification of the ideal cell type; one that promotes neural repair and avoids adverse effects.Specifically, the ideal cell would address issues associated with immunological concerns, the potential for tumorigenesis, ethical issues, lack of cell survival, and limited availability (Banerjee et al., 2011; Clarke and van der Kooy, 2009; Dailey et al., 2013a; Diamandis and Borlongan, 2015; Fisher et al., 2009; Kenmuir and Wechsler, 2017; Polak, 2010; Savitz et al., 2004, 2011a; The Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) Participants, 2009).

To date, studies have used a variety of cell sources including mesenchymal stem cells (MSCs), bone marrow stem cells (BMSCs), and human umbilical cord blood cells (hUCBCs) to promote recovery following stroke (Eckert et al., 2013; Ikegame et al., 2014; Liu et al., 2013; Vendrame et al., 2004; Wei et al., 2013). Neural precursor cells (NPCs) have also demonstrated efficacy in several models of stroke (Bacigaluppi et al., 2008, 2009, 2016; Chang et al., 2013; Chen et al., 2016; Hermann et al., 2014; Liu et al., 2009; Tsupykov et al., 2014). NPCs are comprised of neural stem cells (NSCs) and their progeny and have the capacity to differentiate into neurons, oligodendrocytes, and astrocytes, making them a good candidate for CNS repair strategies.

In our studies, we have used human cells that have been directly reprogrammed from somatic cells to neural precursor cells (drNPCs), without passing through a pluripotent state during reprogramming (Ahlfors, 2016; Ahlfors and Elayoubi, 2010). These cells afford a number of benefits for cell transplantation by overcoming barriers associated with exogenous cell therapy because they are reprogrammed without the use of viral constructs and thus provide a safe and ethically sound source of cells that enable autologous patient-specific cell transplantation, bypassing risks associated with immune rejection and pluripotency (Figure 1). Additionally, these cells may be optimal for cell-based therapies to treat the CNS as the reprogramming technique is more efficient than other methodologies used to generate NPCs (Connor, 2017; Maucksch et al., 2013; Mertens et al., 2016). Together, these highlight the advantage of drNPCs in that they can be taken directly from a patient, produced within a relatively short timeline, and give rise to appropriate neural cell types for CNS repair with high efficiency.

In this study, we explore the therapeutic efficacy of transplanting drNPCs, a clinically relevant and novel population of cells, to treat the stroke-injured brain.
2. Literature Review

2.1 Ischemic Stroke Pathophysiology

Ischemic stroke results in a loss of cells within minutes following onset. On average, for every hour that a stroke is left untreated, a patient loses brain cells and neural function at a rate similar to 3.6 years of normal aging (Saver, 2006). Mechanisms that contribute to the cell loss include excitotoxicity, calcium dysregulation, oxidative and nitrosative stress, cortical spreading depolarization, disruption of the blood-brain barrier (BBB), edema, and inflammation (Deb et al., 2010; Dirnagl et al., 1999; Iadecola and Anrather, 2011; Moskowitz et al., 2010; Rayasam et al., 2017; Woodruff et al., 2011; Xing et al., 2012). These mechanisms propagate the rapid death of all cells within the central core of the ischemic insult, with a slower and more progressive cell death occurring in the surrounding penumbra region, which can remain viable for hours after the initial insult (Figure 2). The slower death rate in the penumbra can be attributed to overlapping blood supply from unaffected collateral arteries (Chavez et al., 2009; Genova, 2011).

The progression of stroke occurs over a span of overlapping phases (Figure 3). In the acute phase following stroke, which lasts up to 4 days, necrotic death in the ischemic core as a result of anoxic depolarization and lack of adenosine triphosphate (ATP) causes irreversible damage within minutes of stroke onset. As the cells within the ischemic core send inflammatory signals, the cells within the penumbra also begin to undergo apoptotic cell death, which amplifies recruitment signals to immune cells within the brain and in the periphery. In this sub-acute stage, starting 48 hours after stroke and lasting up to a month post-stroke, cells release cytokines and chemokines that recruit immune cells towards the ischemic infarct. At this point, the peri-infarct environment and extra-cellular milieu are exposed to a combination of pro- and anti-inflammatory signals until they eventually begin to balance and stabilize the surrounding tissue. It is this stage that most novel therapies aim to target in an attempt to treat stroke. The response to the ischemic infarct subsides around 6 weeks following stroke, which marks the beginning of the chronic phase. Most rehabilitative strategies aim to improve function at this stage.

Following stroke, reactive astrocytes, activated microglia, and infiltrating immune cells contribute to the formation of a glial scar (Burda and Sofroniew, 2014; Huang et al., 2014; Rolls et al., 2009; Silver, 2016; Sofroniew, 2009). Like any scar in the body, the goal of the glial scar is to seal off damaged tissue. Reactive astrocytes along with proteoglycans and other cells (e.g. macrophages) form a dense barrier that surrounds the injured tissue and attempts to prevent
exacerbation of the injury (Silver, 2016; Sofroniew, 2009). The glial scar has long been thought to inhibit axonal growth and tissue regeneration, however, more recent studies suggest that its formation may aid in neurogenesis and NPC activation (Anderson et al., 2016; Cregg et al., 2014; Fitch and Silver, 2008; Liddelow and Barres, 2016; Rolls et al., 2009; Sabelström et al., 2013; Silver, 2016; Silver and Miller, 2004). Taken together, these findings highlight the complexity of the CNS response to stroke and the need to consider these parameters in developing novel therapeutics.

2.2 Current treatment strategies

Therapeutic interventions available to treat stroke have largely focused on blood flow restoration and neuroprotection to prevent cells from dying following the initial insult. These interventions have limited success and those that have been successful are time sensitive, requiring their administration within a short time window following stroke onset for optimal efficacy.

The most common treatment for stroke is the Food and Drug Administration (FDA) approved tissue plasminogen activator (tPA), which breaks down blood clots to restore blood flow in the brain and in turn, prevent further cell loss. However, this approach is limited by the short duration of its maximum therapeutic window (~4.5 hours following stroke) with greatest benefits if administered within the first 2.5 hours of stroke onset (Broussalis et al., 2012; Saver et al., 2015). Other approaches include acute therapeutic hypothermia, physical removal of the clot along with other mechanical treatments, and the administration of aspirin to reduce the likelihood of a secondary stroke, which are still limited by a time-sensitive short therapeutic window.

Currently, there are no FDA approved neuroprotective drugs used in the clinic for stroke. Drugs, such as Edaravone (antioxidant), Ginsenoside-RD (steroid-like compound), and NA-1 (postsynaptic density protein 95 inhibitor), are currently in development and clinical trials but face similar limitations related to the length of their therapeutic window when administration needs to occur within the first 72 hours following stroke (Cook et al., 2012; Hill et al., 2012; Kaste, 2012; Lapchak, 2010; Miyaji et al., 2015; Nabavi et al., 2015; Nakase et al., 2011; Xiao et al., 2007; Zhang et al., 2016).

The only approach currently available to treat chronic disabilities resulting from stroke is rehabilitation. Although this approach has shown some promise, it is time and labour intensive,
can result in variable outcomes, and there is no standardized approach to implementation and evaluation (Wahl and Schwab, 2014; Winstein et al., 2016; Young and Forster, 2007). Brain-machine interfaces, neurostimulation, neuromodulation, and the use of virtual reality have also been shown to improve functional outcomes following stroke, especially when paired with rehabilitation (Corbetta et al., 2015; Kakuda et al., 2011; Laver et al., 2015; Lee and Chun, 2014; Lee et al., 2015; Meinzer et al., 2016; Momosaki et al., 2015; Soekadar et al., 2015; Turolla et al., 2013; Venkatakrishnan et al., 2014). Regardless, the majority of stroke-targeted therapies remain limited in their scope, exemplifying the clear need for novel therapeutic interventions.

2.3 Models of stroke

In order for pre-clinical studies to be effective, appropriate animal models that mimic the human condition are needed. With the knowledge that stroke results in heterogeneous outcomes, the advantages and disadvantages of each model must be considered along with its respective appropriateness for the type of study (e.g. regenerative versus neuroprotective) and the outcome measures to be used (Carmichael, 2005; Fisher et al., 2009; Fluri et al., 2015; Jolkkonen and Kwakkel, 2016; Kumar et al., 2016; Macrae, 2011; Savitz et al., 2011b; Sommer, 2017; The STEPS Participants, 2009). For preclinical therapeutic recovery research, a model that results in predictable, reproducible tissue damage and functional deficits is essential (Diamandis and Borlongan, 2015; Stroke Therapy Academic Industry Roundtable (STAIR), 1999; The STEPS Participants, 2009).

Craniektomy models of stroke, such as pial vessel disruption (PVD) and direct occlusion of cerebral vessels, are performed by removing part of the skull and directly disrupting the cerebrovasculature (Kolb et al., 1997; Sofroniew et al., 1983; Stephens et al., 1985; Tamura et al., 1981). These models provide the benefit that they are easily replicated and lead to consistent and reproducible infarcts. However, these models can affect intracranial pressure and temperature due to the removal of part of the skull and are highly invasive in nature. In addition, PVD models lead to severe neurotrauma as a result of mechanical damage and are thought to better mimic traumatic brain injury (Bartnik et al., 2001; Kleim et al., 2007). Therefore, their translatability in ischemic stroke research may not be optimal for preclinical studies.

The embolic stroke model involves blocking vessels using small spheres made of a variety of different materials or by causing thrombosis (Durukan and Tatlisumak, 2007; Mayzel-
Oreg et al., 2004; Roos et al., 2003; Zhang et al., 1997; Zivin et al., 2009). The location and extent of injury depend on the size of spheres. Microspheres occlude smaller vessels leading to random and unpredictable ischemic insults throughout the brain that vary by location and size (Mayzel-Oreg et al., 2004). Conversely, macrospheres, which block larger vessels, result in a more severe injury that can account for a sizeable portion of a cerebral hemisphere (Gerriets et al., 2003; Walberer and Rueger, 2015). Thromboembolic infarcts require installation of clots that can have significant variability in terms of size and location, but are important for the study of thrombolytic agents (Busch et al., 1997; Jin et al., 2014; Marinescu et al., 2014; Niessen et al., 2003; Zhang et al., 2015). Ultimately, while these models have translational potential, they generate variable ischemic insults which makes them challenging for use in recovery studies.

The most commonly used model of stroke in rodents is the Medial Cerebral Artery Occlusion (MCAO) (Herson and Traystman, 2014; Kumar et al., 2016; Sommer, 2017), which targets the same area that is most often affected in human stroke (Fluri et al., 2015; Kumar et al., 2016; Olsen et al., 1985). This model can be used to produce permanent ischemia by suturing the MCA or to mimic transient ischemia, in which reperfusion is eventually introduced after a given amount of time following removal of the suture (Chiang et al., 2011; Longa et al., 1989). In the transient model, the severity and extent of damage can be controlled by the duration of vessel blockage (Engel et al., 2011; Lee et al., 2014; Morris et al., 2016). This model results in a reproducible ischemic lesion. However, in mice, MACO infarctions are limited because the occlusion tends to affect the majority of the hemisphere, leading to severe damage including components of the subventricular zone (SVZ), cortex, striatum, thalamus, and the hippocampus (Fluri et al., 2015; Lee et al., 2014; Liu and McCullough, 2011). The extent of this damage makes it difficult to identify the source of functional impairment for recovery studies. Although MCAO is a good model of stroke, it may be more representative of complete hemispherical ischemia and provides little benefit in mimicking focal ischemia for localized cell transplant translational studies.

Photochemistry is another methodology used to generate a stroke injury. In this model, specific wavelengths of light are used to activate a photosensitive dye that is injected into the circulatory system, generating a photothrombotic stroke caused by photo-oxidation (Labat-gest and Tomasi, 2013; Li et al., 2014; Rosenblum and El-Sabban, 1977; Watson et al., 1985). The reaction results in oxygen radicals, such as single oxygens and superoxide, that lead to
endothelial damage, platelet activation and aggregation, as well as clotting in blood vessels (Dietrich et al., 1986; Schmidt et al., 2012; Talley Watts et al., 2015; Watson et al., 1985). This model provides a number of advantages including the induction of localized stroke to a desired location, and being highly reproducible. The model, however, is only effective in producing a cortical stroke in which the lesion often does not result in the formation of a penumbra (Labat-gest and Tomasi, 2013) thereby limiting its translational potential.

Endothelin-1 (ET-1) is a potent vasoconstrictor that acts on smooth muscle cells in the vasculature and is commonly used to induce an ischemic insult. Ischemia can be achieved through localized injection into brain parenchyma or direct application of ET-1 onto the surface of the cortex (Gilmour et al., 2004; Horie et al., 2008; Macrae et al., 1993; Roome et al., 2014; Sharkey, 1993; Tennant and Jones, 2009; Wang et al., 2007; Windle et al., 2006). This model of stroke leads to rapid cerebral blood flow reduction followed by reperfusion over several hours (Biernaskie et al., 2001; Gartshore et al., 1996; Macrae et al., 1993) and results in a core ischemic insult surrounded by a penumbral region. The severity of the lesion can be controlled by the concentration and volume of the ET-1 used. The application of ET-1 to induce stroke allows for localized focal lesions that can be easily targeted. However, challenges associated with the use of ET-1 arise because cells in the CNS, such as oligodendrocyte progenitor cells (Gadea et al., 2009), astrocytes (Nakagomi et al., 2000; Rogers et al., 2003), and neurons (Naidoo et al., 2004; Nakagomi et al., 2000) express receptors for ET-1 (ET\textsubscript{A}R and ET\textsubscript{B}R) and endothelin converting enzymes. The effect of ET-1 on these cells may include oligodendrocyte migration and differentiation, astrocytosis, and axonal sprouting, which can affect a variety of processes that contribute to recovery following stroke (Carmichael, 2005; Gadea et al., 2009; Sommer, 2017) highlighting the need to use appropriate controls when using the ET-1 model. Thus, the ET-1 model is effective for use in cell transplantation studies, as it results in a focal, reproducible lesion in targeted areas and can lead to functional deficits.

2.4 Cell Transplant Therapies for the Treatment of Stroke

Stroke is an ideal target for cell based therapies due to the need for therapeutic interventions to replace lost cells, expand the therapeutic window, and more importantly, promote functional recovery (Boltze et al., 2015; Diamandis and Borlongan, 2015; Fisher, 2003; Fisher et al., 2009; Janowski et al., 2015; Lindvall and Kokaia, 2011; Meamar et al., 2013; Savitz
et al., 2011a; The STEPS Participants, 2009). Several studies suggest that stem cell transplants can be beneficial and promote recovery following stroke (Bacigaluppi et al., 2008; Banerjee et al., 2011; Chen et al., 2016; Hermann et al., 2014; Janowski et al., 2015; Meamar et al., 2013; Napoli and Borlongan, 2016; Rosado-de-Castro et al., 2013; Wang et al., 2017). However, a number of limitations exist, including the identification of the optimal cell type.

2.4.1 Cell Types and Sources

Stem cells have two cardinal properties; they can self-renew and they are able to proliferate and give rise to progeny which can differentiate into mature tissue-specific cells. Stem cells can be totipotent, pluripotent, multipotent, bipotent, or unipotent. Totipotent cells are able to give rise to all cell types, including extra-embryonic cells, such as those found in the placenta. Pluripotent cell populations include embryonic stem cells (ESCs), which are derived from the inner cell mass of the developing blastocyst, and induced pluripotent stem cells (iPSCs), which are somatic cells that were originally reprogrammed by means of four inducible factors (Oct3/4, a homeodomain transcription factor involved in self-renewal of stem cells and pluripotency; Sox2, a SOX transcription factor involved in maintenance of stem cells, especially NPCs and plays important roles in embryogenesis; c-Myc, a nuclear phosphoprotein that functions as a transcription factor involved in cell cycle progression; and Klf4, a zinc-finger transcription factor involved in cell cycle control as well as proliferation and differentiation) using viral integration (Takahashi and Yamanaka, 2006). ESCs and iPSCs give rise to cells comprising all 3 germ layers but can be specifically differentiated, using well defined protocols, into multipotent cells from specific lineages (Di Bernardini et al., 2014; Kogut et al., 2013; Lian et al., 2016; Mallanna and Duncan, 2013; Morizane and Lam, 2015; Schwartz et al., 2008; Xu et al., 2013). Adult multipotent cells, such as MSCs, can be found in a variety of tissues and give rise to multiple tissue specific cells. Bipotent stem cells, such as hepatic oval cells, have the ability to only give rise to two different cell types, whereas unipotent cells, like spermatogenic stem cells, can only differentiate into a single specific cell.

Most cell-transplant research for stroke has focused on adult multipotent stem cells, such as MSCs, which can be found in multiple tissues; BMSCs, found in the bone marrow; and NPCs, which reside in the brain (Bang, 2016, 2016; Doeppner and Hermann, 2014; Gutiérrez-Fernández et al., 2012; Hermann et al., 2014; Rosado-de-Castro et al., 2013). Transplanting
pluripotent and multipotent cells for the treatment of stroke in animal models has resulted in recovery, albeit at varying degrees (Doeppner and Hermann, 2014; Modo et al., 2002; Wu et al., 2015; Yang et al., 2016). It has been speculated that non-neural cells aid in stroke recovery by providing trophic support, inducing neuroplasticity, modulating the inflammatory immune response, or activating endogenous stem cells (Baraniak and McDevitt, 2010; Gutiérrez-Fernández et al., 2012, 2013; Horie et al., 2015; Janowski et al., 2015; Satani and Savitz, 2016; Sullivan et al., 2015; Wei et al., 2017). Yet, these cells lack the ability to replace the lost brain tissue that results from stroke.

Tissue specific stem cells are limited to the generation of cells within their tissue of origin. Accordingly, CNS cell replacement strategies use brain and spinal cord derived neural precursor cells that are neurally committed (Gage and Temple, 2013). NPCs, a population comprised of NSCs and their progeny, can differentiate into neurons, oligodendrocytes, and astrocytes. Multiple studies have proven the efficacious and beneficial effects of transplanting NPCs to treat stroke that result in improved outcomes, such as better functional performance, decreased glial scarring, and diminished extent of injury, without resulting in tumor formation (Bacigaluppi et al., 2009; Ballios et al., 2015; Chang et al., 2013; Gervois et al., 2016; Liu et al., 2009; Mohamad et al., 2013). The mechanism(s) by which the cells provide benefit is thought to be multifaceted, as it there is evidence that NPC transplants not only integrate into the brain tissue and become part of the circuitry, but they can also provide trophic support, activate endogenous stem cells, and modulate the inflammatory response to stroke (Baraniak and McDevitt, 2010; Drago et al., 2013; Hermann et al., 2014; Satani and Savitz, 2016). A meta-analysis and systematic review examining the role of NPC transplants for stroke confirmed that transplanting NPCs improved functional and structural outcomes with significant effect sizes (Chen et al., 2016). The use of NPCs appears to be a promising avenue for the development of novel treatment strategies for stroke.

2.4.2 Considerations for Cell Selection

A number of concerns and obstacles surrounding cell-based therapeutic interventions exist. The possible risks and dangers associated with regenerative medicine could result in a failed intervention, or worse, exacerbate the damage with the development of a slew of complications such as tumors and adverse effects like seizures or worsening of symptoms
Apart from these effects, considerations for stem cells to be used as a therapeutic intervention should also account for immunorejection, pluripotency, ethics, and the availability of the cell source.

2.4.2.1 Immunorejection
The source of cells has many implications on the success of an intervention. Xenogenic cells are derived from a different species; allogenic cells come from a donor of the same species; syngeneic cells can be sourced from an identical twin; and autologous cells come from the patient directly. Cells that do not come from the host (or are not genetically identical to the host) will be recognized as a foreign body by the host immune cells and will be subject to an immune response. Once the recognition of foreign cells occurs through the identification of antigens on the major histocompatibility complex (MHC), T-cells become activated and expand, leading to the production and recruitment of cells than can directly or indirectly destroy the transplanted foreign cells (Ingulli, 2010; Issa et al., 2010). This immune response can lead to a cascade of inflammatory responses that cause further tissue damage (Fainstein et al., 2013; Ingulli, 2010; Issa et al., 2010; Jin et al., 2013; Rayasam et al., 2017). Clearly, the immune response to cell transplant can play a vital role in the success of a treatment strategy.

2.4.2.2 Pluripotency and Teratoma formation
Pluripotent cells, such as ESCs and iPSCs, carry with them the risk of tumorigenesis, resulting in the formation of teratomas and teratocarcinomas (Gervois et al., 2016; Kawai et al., 2010; Ye et al., 2013). Teratomas are benign tumors containing cells from all three germ layers with normal karyotypes, while teratocarcinomas are aggressively malignant tumors that contain highly proliferative undifferentiated cells. Induction of iPSCs using viral or genetic modification reprogramming methods carries the risk of mutagenesis and genotoxicity posing a source for caution because such alterations in the genetic material can lead to abnormal outcomes like apoptosis and tumor formation as a result of dysfunctional or dysregulated mRNA (Clarke and van der Kooy, 2009; Hong et al., 2013). Incomplete reprogramming or contaminated cell cultures can also lead to aberrant results following iPSC transplantation (Gervois et al., 2016; Hong et al., 2013; Wei et al., 2017). As such, the therapeutic potential of pluripotent cells is
challenged by the fact that their differentiation is more difficult to control than NPCs and there is a higher risk of forming teratomas.

2.4.2.3 Ethical Concerns
Ethical and moral concerns are another consideration that must be made when selecting appropriate candidate cells for stroke recovery. Ongoing debates surrounding ESCs and fetal stem cells have made research difficult to conduct and clinical application even more challenging. The issue raised with these cell types is the ethical implication of sourcing the cells in order to transplant them, as it requires harvesting cells from human embryos and fetuses.

2.4.2.4 Availability of Cells
A number of different cell types can be harvested from multiple sources for the use of therapeutic transplant interventions. Procedures for isolating stem cells range in their level of invasiveness, the type of cell being collected, and the number of cells yielded for transplant. Cells to be used in transplantation can be harvested from a number of tissues including bone marrow, synovium, periosteum, adipose tissue, muscle, blood, brain, skin, and embryo, amongst others (Bang et al., 2016; Dailey et al., 2013b; Elias et al., 2015; Sakaguchi et al., 2005; Shinozuka et al., 2013; Vishwakarma et al., 2014). Although there is some evidence that non-neural precursors can differentiate to neural cell types (Fernandes et al., 2006; Fu et al., 2008; Jang et al., 2010; Joannides et al., 2004; Kucia et al., 2006; Mezey et al., 2000; Sanchez-Ramos, 2002; Toma et al., 2001; Wislet-Gendebien et al., 2005; Yu et al., 2014), NPCs are still considered the most appropriate cell source to regenerate neural tissue without the production of other tissue types. The challenge with their use is the difficulty in harvesting them from the mature CNS.

Neural stem and progenitors cells are found in the SVZ lining the lateral ventricles in the adult forebrain. Following stroke, endogenous NPCs are activated in vivo and migrate to the lesion site but the magnitude of their response is sub-therapeutic (Dibajnia and Morshead, 2012). Generating neural stem cells for transplantation consists of (1) removing cells from embryonic or fetal brain tissue (2) directly collecting them from the adult CNS or (3) reprogramming somatic cells through an inefficient processing regime that yields low results (Gervois et al., 2016; Kenmuir and Wechsler, 2017; Mertens et al., 2016). Isolating NPCs from adults for autologous transplants is a difficult process, sourcing cells derived from embryonic or fetal neural tissue can
be a source of ethical concern, and generating iPSCs and differentiating them to a neural lineage is a time-consuming process that requires many weeks to complete. None of these approaches are optimal for clinical therapies. With such limited availability and accessibility, translating basic research to clinical practice using human cells poses a serious challenge.

2.4.3 Reprogramming Cells to NPCs

There are three sources from which to derive autologous adult NPCs; directly from the adult CNS, by reprogramming mature somatic cells to iPSCs and then differentiating them to the neural lineage, or directly transdifferentiating them to NPCs without going through a pluripotent state.

When considering the use of iPSCs for cell transplantation, the benefits include the fact that they are an autologous source of cells that can circumvent immune rejection. However, transplantation of iPSCs remains controversial because of the known risk of teratoma formation and the potential to differentiate into undesirable cell types post-transplantation (Hong et al., 2013; Kawai et al., 2010; Ye et al., 2013). To minimize the risk, iPSCs can be pre-differentiated into a desired cell type prior to transplantation, although demonstrating the complete absence of pluripotent cells prior to transplanting is difficult (Choi et al., 2014; Fu et al., 2012). In addition, the length of time to reprogram iPSCs and subsequently differentiate them into the appropriate neural lineage is lengthy, and thus may not be suitable for clinical applications.

Direct cell reprogramming, otherwise known as transdifferentation, occurs when one somatic cell is directly transformed into a different cell type without going through a pluripotency stage. A variety of methods have been used to directly reprogram somatic and non-neural stem cells to neurons or NPCs, including viral transduction of a combination of multiple different factors (Cassady et al., 2014; Lujan et al., 2012), a combination of Brn4/Pou3f4, Sox2, Klf4, c-Myc, and E47/Tcf3 (Han et al., 2012), Ascl1, Brn2, and Myt1l (Vierbuchen et al., 2010), only Sox2 and c-Myc (Castaño et al., 2014), and even Sox2 alone (Ring et al., 2012). One way to reprogram cells without viral vectors is using a plasmid or by means of a synthetic mRNA gene delivery system that transfects fibroblasts with PAX6 and SOX2, reprogramming them to neurons within 14 - 28 days (Connor, 2017; Jones, 2017; Mccaughey-Chapman, 2017) In the reprogramming paradigm used for our cells, somatic cells were isolated and transformed via transient expression of Musashi-1 (Msi1), a neural RNA-binding protein that contributes to self-
renewal of NSCs through the notch signaling pathway (Okano et al., 2005); Neurogenin-2 (Ngn2), a helix-loop-helix transcription factor that can be used to reprogram cells and induce differentiation to mature neurons (Hand and Polleux, 2011; Hand et al., 2005; Thoma et al., 2012); and Methyl-CpG Binding Domain Protein 2 (MBD2), a protein that regulates neural differentiation and gene expression of cells through an HDAC-dependent epigenetic mechanism (Díaz de León-Guerrero et al., 2011; Fan and Hutnick, 2005); generating drNPCs within 2 weeks of transfection. This paradigm results in cells that have the characteristics of human NPCs, including the ability to form neurospheres and generate neural progeny, without the formation of tumors, teratomas, or non-neural tissue (Ahlfors, 2016; Ahlfors and Elayoubi, 2010; Azimi, 2016; Khazaei, 2016, 2017; Vonderwalde, 2017). The reprogramming protocol efficiently produces a higher yield of drNPCs in less time than other methodologies (Hu, 2014; Mertens et al., 2016). Additionally, drNPCs do not show evidence of tumorigenesis following in vivo transplantation (Azimi, 2016; Khazaei, 2016, 2017). Accordingly, these cells adequately meet criteria outlined for cellular approaches to treating stroke (Diamandis and Borlongan, 2015; Lapchak et al., 2013; Stroke Therapy Academic Industry Roundtable (STAIR), 1999) making them an interesting and relevant cell population to investigate.

2.4.4 Transplant Parameters for Neural Precursor Cells
While increasing evidence points to the efficacy of using NPCs to treat stroke, the optimal transplant paradigm has yet to be determined. A number of factors such as: (i) the timing of the transplant post-stroke, (ii) the dose of cells transplanted, (iii) the method of administration, and (iv) the delivery vehicle used for transplantation can influence transplant success. Optimization of these parameters is critical for clinical translation of cell-based therapy to treat stroke.

2.4.4.1 Timing of Transplantation
The aim of transplanting NPCs can be to (1) replace lost cells, (2) release of factors that modify the microenvironment and/or (3) promote cell survival of host cells and/or (4) promote neuroplasticity of the host tissue. The time of cell transplantation following stroke is strongly related to the success of the intervention as measured by functional and structural outcomes (Chen et al., 2016). It appears that transplanting cells within the sub-acute phase of stroke leads to the greatest outcomes. Indeed, rats that received NPC transplants derived from embryonic
brains (E14) directly into the ipsilateral lateral ventricle following MCAO demonstrated the most improvement when cells were transplanted 3 days following stroke (compared to 1 hour, 12 hours, 1 day, 5 days, and 7 days) as defined by smaller infarct size, reduced apoptosis, and improved behavioural recovery (Ziaee et al., 2017). Similarly, human fetal striatum NPCs transplanted into the rat striatum following MCAO exhibited greater survival and increased neural differentiation at 48 hours compared to those transplanted 6 weeks following stroke (Darsalia et al., 2011). Interestingly, NPCs isolated from adult mouse SVZ that were transplanted into a mouse model of ischemia lead to a reduction of brain injury if transplanted at 0 or 1 days post-stroke compared to 28 days post stroke, but cells transplanted 28 days after stroke had greater survival and engraftment compared to those transplanted at early time points (Doeppner et al., 2014). Functional recovery was reported regardless of the transplant time, indicating that cells may act through a variety of mechanisms to promote recovery. These studies show that the use of NPC transplants to treat stroke has the potential to expand the therapeutic window. When considering the success of transplant strategies for optimal outcomes, the consensus appears to be that cells should be transplanted in the sub-acute stage, ideally within 72 hours following stroke in animal models (Chen et al., 2016). Thus, in order to promote beneficial effects following stroke, the optimal timing for transplantation in rodent models may be between 2 – 4 days following stroke. Interestingly, this timeframe coincides with the end of the initial inflammatory response (near the early stages of the sub-acute phase), when the tissue is more permissive to transplant survival but still susceptible to modulation.

2.4.4.2 Transplantation Dose

The cell dose is another parameter that can affect the success of the transplant. Few studies that have investigated the effect of varying the NPC dose on transplant success by specifically examining how the number of cells transplanted can influence overall cell survival, proliferation, and functional recovery. It is difficult to compare data from different studies, as the dose is not the only thing that changes; the timing, the location, and even the model plays a role in transplant outcomes. As such, only those studies that have investigated the dose response of NPCs can be used to accurately predict the impact of dose on transplant success. There are conflicting reports on how transplant dose of human NPCs affects recovery and the survival of transplanted cells. One study indicated that increasing human NPC dosage leads to increased cell
survival (Piltti et al., 2015), while others have reported an inverse relationship in that increased NPC transplant dose (750,000 and 1,500,000) results in a lower percent of cells surviving than when 300,000 cells were transplanted (Darsalia et al., 2011). Interestingly, human NPC transplants indicate that using a ‘medium-dose’ (45,000 cells) results in greatest chance of survival compared to low dose (4,500 cells) and high dose (450,000 cells) transplants (Stroemer et al., 2009). In another study the absolute number of surviving cells was not significantly different between dosage groups (Darsalia et al., 2011). It is possible that these results vary due to the source of cell used, the transplant paradigm, and the timing of transplant or sacrifice following injury. In terms of cell proliferation once transplanted, there seems to be an inverse relationship between transplant dose and the number of transplanted cells in the brain (Stroemer et al., 2009) and spinal cord (Piltti et al., 2015) at time of sacrifice; the more cells that are transplanted, the less proliferation. It is possible that proliferation is dependent on growth factors and cells within denser transplants have more difficulty accessing nutrients. Thus, there may be a certain threshold of cells to transplant, limited by insufficient resources.

When relating the dosage to behavior recovery, one study reports no significant correlations between behavioural performance and the number of surviving cells, yet those animals that received a greater number of transplanted cells exhibited the greatest improvement (Stroemer et al., 2009). Conversely, a meta-analysis conducted on NPC transplantation for preclinical stroke studies reports that the transplant dose itself does not seem to be relevant in order to produce functional benefits (Chen et al., 2016). Similarly, increasing the dose of NPCs does not result in significant reduction in lesion volume (Stroemer et al., 2009) or provide structural benefits (Chen et al., 2016). This further supports the notion that human NPC transplants may be acting through mechanisms other than integration and cell replacement.

2.4.4.3 Methodologies for Cell Delivery

NPCs can be transplanted through several different routes (Willing and Shahaduzzaman, 2013), though few studies have directly compared them. Most commonly, cells are administered via intravascular or intracerebral methods. In both methods of delivery, benefits have been observed related to functional recovery.

Intravascular delivery can be accomplished intra-arterially and intravenously. Findings suggest the effects of intravascular injection may be due to trophic support provided by the
transplanted cells as little cell engraftment occurs using this method (Doeppner et al., 2015). Intravenously transplanted cells are commonly found in peripheral tissues and result in aberrant engraftment with fewer cells reaching the brain. In contrast, intra-arterial delivery bypasses peripheral organs, allowing more cells to reach the brain and limiting the numbers of cells found in peripheral tissues (Li et al., 2010). Risks associated with intra-arterial delivery, however, include a greater possibility of microemboli formation and the associated increased risk of mortality (Doeppner et al., 2015; Li et al., 2010; Rodríguez-Frutos et al., 2016).

Intracerebral transplants are the most common and invasive approach. This surgical approach to deliver cells directly to the brain parenchyma results in the most precise placement of the cells and the greatest number of engrafted cells (Bliss et al., 2010; Doeppner et al., 2015; Liu et al., 2014). Cells can be grafted directly to the injury site or in a surrounding location (proximal or distal to the lesion and/or penumbra) from which they can migrate to the injury site (Jin et al., 2005). Apart from migrating to the lesion, cells can promote recovery via mechanisms other than cell replacement, such as providing trophic support and enhancing neuroplasticity (Andres et al., 2011). The risk, however, is that cells do not migrate and thus reside in the surrounding parenchyma as an aggregate capable of damaging or changing the dynamics of an uninjured brain region (Bliss et al., 2010). Since the ischemic region is considered a hostile environment for transplanted cells, in particular at early times post-stroke, it is important to develop a strategy to promote survival of transplanted cells. This has been successfully achieved by preconditioning cells to hypoxic environments, co-transplanting cell with trophic factors for support, or using biomaterials (Cooke et al., 2010; Donaghue et al., 2014; Sart et al., 2014; Yu et al., 2013). Even when considering hurdles that intracerebral transplantation poses, this method is considered optimal for getting cells to the brain for cell replacement.

Intranasal transplant strategies have recently emerged as a novel and promising approach to cell delivery, although to date, this methodology has not been used with NPCs for stroke. Success has been reported, however, in stroke with non-neural cells and with NSCs delivered to treat other brain disorders such as intracerebral gliomas, Parkinson’s Disease, and a model of multiple sclerosis (Danielyan et al., 2011; Reitz et al., 2012; Wei et al., 2013; Wu et al., 2013). With this minimally invasive strategy, cells enter the brain parenchyma without passing through systemic vasculature allowing for easier access to the injury site (Chapman et al., 2013;
Danielyan et al., 2009). This promising deliver strategy is an important avenue to pursue in future studies, as evidence of its efficacy is still limited.

### 2.4.4.4 Injectable biomaterials as a scaffold for NPCs

Bioengineered vehicles, such as hydrogels and scaffolds, are important considerations for transplants into the stroke lesion site. Several vehicles have been explored to date with the goal of promoting cell survival and integration (Cooke et al., 2010; Marquardt and Heilshorn, 2016; Nih et al., 2016). Biomaterials provide the benefit of a supportive microenvironment that allows transplanted cells to survive when transplanted into a hostile ischemic atmosphere. Ways to further improve viability include embedding adhesion molecules and growth factors directly within the hydrogel. These modifications to the biomaterial can enhance cell survival by addressing two of the main causes of apoptosis following transplantation: detachment from a matrix (anoikis) and limited access to growth factors (Cooke et al., 2010). Besides providing a niche conducive for cell survival, biomaterials also prevent aggregation of cells and can modulate inflammatory responses to injury that lead to necrosis and cell death (Ballios et al., 2010; Cooper et al., 2008; Gupta et al., 2006; Nih et al., 2016; Singh and Peppas, 2014; Vishwakarma et al., 2016; Wang et al., 2012b; Zhong et al., 2010). The use of hyaluronan methylcellulose (HAMC) hydrogel as a transplant vehicle has resulted in improved NPC viability, cell distribution, and functional recovery in mouse models of stroke (Ballios et al., 2015), retinal degenerative diseases (Ballios et al., 2010), and spinal cord injury (Mothe et al., 2013). In addition to promoting cell survival in vitro and in vivo, HAMC has two key properties that facilitate transplantation; the hydrogel is inverse thermal gelling, meaning it will only gel once it is in the body, and shear thinning, allowing for easy injection (Caicco et al., 2013; Shoichet et al., 2015). Utilization of biomaterials that diminish the harsh conditions cells face following transplantation, yet still allow for cell survival and differentiation are proving to be an encouraging avenue to pursue in the development of regenerative therapies following CNS injury.

### 2.5 Outcome Measures

When considering the efficacy of a novel approach, cell/tissue analysis and functional outcomes are used as measure of success. Arguably, behavioural outcomes are the most clinically relevant measures.
2.5.1 Functional Analysis

In sensorimotor mouse models of stroke, a variety of different behavioural tasks can measure functional performance and recovery. As such, it is necessary to utilize a stroke model that results in persistent long-term deficits that do not spontaneously recover. The model of choice and the mouse strain may play a role in the prognosis of the experimental stroke deficits. Indeed, previous work in our lab has shown that immunocompromised NOD/SCID mice spontaneously recover by 25 days following PVD stroke compared to immunocompetent NOD/LTJ control mice (Erlandsson et al., 2011). To elucidate whether an intervention in question provides a therapeutic benefit, it is crucial that untreated animals remain disabled long-term.

The foot fault test, also known as the grid-walking test, has consistently been used to show unilateral motor deficits in various models of mouse stroke (Ballios et al., 2015; Erlandsson et al., 2011; Lourbopoulos et al., 2008; Sachewsky et al., 2014; Schaar et al., 2010; Zhang et al., 2002). This test assesses gross sensorimotor function and balance as it requires mice to use their paws as they explore an elevated grid. The advantage of this test is that it is relatively easy to implement, requires little training, and allows for direct visualization of deficits.

The cylinder test is another assay commonly used to assess motor outcomes, measuring paw use preference during rearing, which is a commonly affected outcome in models of stroke (Brooks and Dunnett, 2009; Schaar et al., 2010; Wu et al., 2015). Control, uninjured animals use both forepaws equally during rearing, whereas those that received a unilateral stroke prefer to use their ipsilateral (non-injured) paw. The cylinder test is easy to implement and score, but has been used with greater success to identify functional deficits in rats and in mice with severe lesions, as small cortical infarcts may not result in a deficit and/or mice may recover spontaneously (Balkaya et al., 2013). Furthermore, this test requires mice to be motivated to perform rearing behaviours, which may be difficult in some models.

Fine sensorimotor functionality can be measured using the tape test, also known as the adhesive removal test (Bouët et al., 2007, 2009; Freret et al., 2009; Nusrat, 2015; Venna et al., 2014). In this test, the elapsed time that it takes a mouse to contact and remove a small piece of adhesive tape from their forepaw is measured and can be used as an indicator of sensorimotor function with impaired animals taking longer to complete the task when the tape is placed on their contralesional paw. This test is beneficial in that it measures both sensory and motor...
function. However, this test is disadvantageous in that it takes multiple training sessions to achieve a plateau in performance and results can vary on the anxiety level of the mice and their willingness to perform the task on the day of testing.

Using a battery of multiple tests increases the probability of detecting motor impairment and functional recovery in at least one assay, which is beneficial when inducing a small cortical infarct. Hence, while each test offers advantages, a combination of more than one test is desirable for motor recovery studies.

2.5.2 Cell and Tissue Analysis
To better understand the underlying source of recovery, the cell and tissue response can be examined. Correlations between these outcomes and functional recovery can provide a basis of support to further understand the role that cells play in recovery and develop more specialized therapeutic interventions.
3.0 Hypothesis and Objectives

3.1 Brief Project Overview
Stroke is one of the leading causes of disability worldwide leading to devastating impairments and decreased quality of life. Cell transplant therapies have demonstrated potential to be a long-term solution to repair the brain following stroke. We have access to a novel population of NPCs that have been directly reprogrammed from somatic cells and have the ability to regenerate neural brain tissue. These drNPCs are clinically relevant as they afford the benefit of using patient specific somatic cells and directly reprogramming them without the use of viral constructs, resulting in a safe and ethically sound source of autologous cells. In this study, we investigated the therapeutic potential of drNPCs in an immunocompromised mouse model of focal ischemia using artificial cerebrospinal fluid (aCSF) or a HAMC hydrogel as a delivery vehicles (Figure 4). The main objective of this project is to explore the potential of human drNPCs to promote tissue repair and functional recovery in a mouse model of stroke.

3.2 Hypothesis
The transplantation of human drNPCs into the stroke-injured brain will promote tissue repair and functional recovery following a cortical focal ischemic insult in an immunocompromised mouse model of stroke.

3.3 Aims
To test our hypothesis, we explored three research aims.

Aim 1: Characterize drNPCs at time of transplantation
Immunohistochemistry, polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and quantitative RT-PCR (RT-qPCR) were used to evaluate the lineage potential of drNPCs and their properties at the time of transplantation in vitro. We predicted that drNPCs would not express pluripotency markers and would be neurally restricted.

Aim 2: Develop and characterize the ET-1 model of stroke in SCID/Beige mice
Immunocompromised SCID/Beige mice (lacking B & T cells; dysfunctional NK cells) received a focal ET-1 stroke in the sensorimotor cortex similar to what has previously resulted in observable behavioural deficits and tissue damage in wild-type mice (Roome et al., 2014;
Sachewsky et al., 2014; Tennant and Jones, 2009; Wang et al., 2013). The model of stroke was evaluated using a combination of behavioural assays and tissue analysis to measure damage in the cortex. We predicted that this model of focal ischemia would result in observable behavioural deficits as well as an ischemic injury and scar formation that permits evaluation of tissue repair and functional recovery following cell transplantation.

**Aim 3: Investigate the therapeutic relevance of drNPC transplants in two vehicles for stroke recovery**

We built on previous studies (Ballios et al., 2015) indicating that cell transplantation on day 4 post stroke, in the presence of HAMC, and at the site of the lesion provided the best parameters for cell survival and functional recovery with mouse NPCs. For our study, SCID/Beige mice were separated into four groups (aCSF alone; HAMC alone; aCSF+drNPCs; or HAMC+drNPCs) for transplantation following stroke (Figure 4A). Functional performance was measured for one month, at which time animals were sacrificed and tissue analysis was performed to examine cell survival and lesion volumes (Figure 4C). We predicted that animals receiving drNPCs would have improved outcomes compared to vehicle controls.
4. Materials and Methods

Study Design

A total of 13 (9 included, 4 excluded) animals were used to establish the stroke model, which was an adequate sample size with an effect size of $f=0.5$, $\alpha$ error probability of 0.05, and a power of 0.95. For transplant experiments, a total of 10 – 14 animals were used per group; an appropriate number of samples to approximate an effect size of $f=0.25$, with an $\alpha$ error probability of 0.05 and a power of 0.95 as calculated using G*Power (Version 3.1). The experimental design was a controlled laboratory experiment that measured both functional recovery and tissue repair following stroke using cellular transplants. Animals were separated into groups via random assignment by a blinded third party until appropriate numbers of samples were achieved for each group. Behavioural analysis was conducted by an observer blinded to the treatment groups.

Cellular analysis (survival and proliferation) following transplant and lesion size and gliosis response post-stroke were conducted by two different observers. Based on our sample sizes, we could detect an effect size of $f=0.63$, with an $\alpha$ error probability of 0.05, and a power of 0.80 when calculating cell survival, and an effect size of 0.73, with an $\alpha$ error probability of 0.05, and a power of 0.80 when calculating cell proliferation in vivo. Lesion volume measurements required an effect size of 0.60, with an given $\alpha$ error probability of 0.05, and a power of 0.80. Gliosis analysis yielded results able to detect an effect size of 0.66, with an $\alpha$ error probability of 0.05, and a power of 0.95. Analysis was conducted using G*Power (Version 3.1).

Ethical Statement

All experimental protocols were in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council of Animal Care and approved by the animal care committee at the University of Toronto.

Animals

Immunocompromised Fox Chase SCID/Beige (8–16 weeks old; CB17.Cg-Prkdc\textsuperscript{scid}Lyst\textsuperscript{bg-}J/Crl; Charles River Laboratories, Wilmington, MA) mice were singly housed on a 12-hour light/dark cycle with food and water provided ad libitum for the duration of testing, starting 3 days prior to stroke, and lasting up to 32 days following stroke. A total of 87 animals
(establishment of stroke, n=13, [sex not tracked]; long-term deficit analysis, n=13 [7 males, 6 females]; confirmation of stable measures in long term testing of naïve animals, n=8 [6 males, 2 females]; therapeutic evaluation, n=53 [30 males, 23 females]) were used in this study.

**Exclusion Criteria**

Outliers were removed by robust regression and outlier removal (ROUT) method on Prism GraphPad (Q=1.0%) based on foot fault performance following stroke and prior to treatment. The mean and standard deviation (STDEV) for baseline performance was calculated for all mice receiving a stroke involved in the therapeutic evaluation and establishment of long-term deficits. Any animal that did not show a deficit (in % Fault Difference) greater than 2 STDEV from the baseline mean up to 8 days following stroke was removed from the study. A total of 1 outliers were removed and 10 animals were excluded due to failure to meet the exclusion criteria (long-term deficit, n=4; therapeutic evaluation, n=6). When exploring functional performance using the cylinder test, we first confirmed normality using the Shapiro-Wilk Normality Test (confidence interval = 95%), then identified outliers by using a whisker and boxplot analysis (SPSS) and Grubb’s Method (Prism Graphpad, alpha = 0.05). A total of 1 outlier from the stroke group was excluded from our analysis.

**Ischemia induction for stroke injury model**

Based on previous work (Ballios et al., 2015; Faiz et al., 2015; Sachewsky et al., 2014) we have demonstrated that ET-1 injections into the sensorimotor cortex of immunocompetent mice resulted in tissue damage and functional impairment following stroke. We utilized this model of injury in an immunocompromised mouse model to enable the transplantation of human derived precursors and avoid immuno-rejection. To induce a focal ischemic insult in the sensorimotor cortex, mice were anaesthetized with isoflurane (5% induction, 1 – 2.5% maintenance), treated with Ketoprofen (0.1ml/10g of body weight; 0.5mg/ml in dosage), and prepared for stereotaxic surgery. An incision was made at the top of the head to expose the skull and drill a small burr hole at the site of the right sensorimotor cortex, located +0.6 mm anterior and -2.25mm lateral to bregma. Mice received a 1µL injection of ET-1 (Calbiochem, 800 picomolar) 1 mm deep from the surface of the brain at a rate of 0.1µL/min using a 2.5µL Hamilton Syringe with a 26 gauge, 0.375” long needle (Hamilton, Reno, NV). The needle was kept in place for 10 minutes following the injection to prevent backflow and then slowly
withdrawn. The incisions were sutured and mice were set to recover under a heated lamp. Naïve controls did not undergo surgery.

**Maintenance and expansion of drNPCs**

Human drNPCs were cultured and expanded on Corning® CellBIND® culture dishes (Corning, Product #2394 to #3296, Tewksbury, MA). Cells were cultured as a monolayer in low oxygen conditions (at 5% CO₂; 5% O₂ and 37°C) in Human NeuroCult XF medium (StemCell Technologies) supplemented with epidermal growth factor (EGF) [20 ng/ml] (Peprotech), fibroblast growth factor-2 (FGF-2) [30 ng/ml] (Peprotech), and heparin [100 µg/ml] (Scientific Protein Laboratories). Cells were detached using Accutase (Innovative Cell, Technologies, Inc. Cat #AT-104) and either passaged or used for transplants once they reached ~80% confluency, occurring within 5 – 7 days of culturing. Cell feeding occurred every 36 hours by replacing 50% of the media.

**HAMC preparation**

The HAMC hydrogel was prepared using a blend of hyaluronan (HA, 1400-1800 kDa, Novamatrix, Drammen, Norway) and methylcellulose (MC, 300 kDa, Shin-Etsu, Tokyo Japan). Sterile-filtered HA and MC were dissolved in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM D-Glucose, 2 mM MgCl₂, and 10 mM CaCl₂ dissolved in double distilled H₂O) at a concentration of 1% HA (w/v) and 1% MC (w/v) producing a final solution of 1/1 HAMC. The 1/1 HAMC was then mixed in a SpeedMixer (DAC 150 FVZ, Siemens) for 30 seconds at 3,500 RPM, centrifuged for 1.5 minutes, and placed on a shaker at 4°C for 14 hours. The following day, once the solution was fully dissolved, the 1/1 HAMC hydrogel was mixed again for 30 seconds at 3,500 RPM using the SpeedMixer, centrifuged for 5 minutes, and stored on ice for 20 minutes. Centrifugation for 5 minutes and storage on ice for 20 minutes were repeated until no bubbles remained in the hydrogel solution. Following, drNPCs in aCSF were added to the 1/1 HAMC in order to dilute it 25% and achieve a final concentration of 0.75/0.75 HAMC. The mixture was then slowly pipetted up and down using a wide orifice pipette tip, and the resulting suspension of drNPCs in HAMC was stored on ice.
**Cell transplantation**

For transplantation, drNPCs were suspended in aCSF, or 0.75/0.75%wt HAMC hydrogel at a concentration of 100,000 cells/µL. Cells were transplanted into the stroke site with the same surgical procedures used for ET-1 induced ischemia in the sensorimotor cortex, as described above. Briefly, the skull with the burrhole was exposed and 1µL of cell suspension (100,000 cells) was injected into the same location as the ET-1 injection 4 days prior at a rate of 0.1µL/min using a 2.5µL Hamilton Syringe with a 26 gauge, .375” long needle (Hamilton, Reno, NV). The syringe was removed 10 minutes following injection to prevent backflow. The incision was sutured, and mice were allowed to recover. Control animals received 1µL injections of aCSF or HAMC only. Transplants using aCSF were carried out within 2 hours of cell suspension, whereas transplants using HAMC were carried out within 6 hours of cell suspension.

**Tissue and cell processing**

Mice were anaesthetized with the use of Avertin (Sigma Aldrich). A transcardial perfusion with cold 0.01M phosphate buffered saline (PBS) solution followed by cold 4% paraformaldehyde (PFA) solution (pH=7.4) was used to fix tissues for analysis. Brains were extracted and placed in 4% PFA for 3 – 6 hours and then transferred to a 20% sucrose in 0.01M PBS solution to be left in overnight. Brains were cryosectioned at 20µm along the coronal plane and collected in series on Superfrost Plus Microscope Slides (Cat #12-550-15, Fisherbrand).

For cell work, *in vitro* sister cell cultures of drNPCs were fixed at the same time that transplants occurred (following 5 – 7 days of culturing in growth factors) using 4% PFA solution (pH=7.4). Cells were then washed in PBS solution.

**Immunostaining**

Fixed tissue and cells were rinsed with 1XPBS, permeabilized with 0.3% Triton-X100 in 0.01M PBS for 20 min and then blocked in 10% Normal Goat Serum (NGS) with 0.3M glycine for 1 hour at room temperature. Samples were then treated with appropriate primary antibodies (*Table 1*) in 0.01M PBS and left overnight at 4°C in a humid environment. Samples were then washed and treated with appropriate secondary antibodies (*Table 2*), followed by a 1 hour incubation at room temperature. The samples were then washed again, cover-slipped with either PBS or mowiol® 4-88 (Sigma-Aldrich), and imaged using an AxioVision Zeiss UV microscope.
or Olympus FV1000 confocal point-scanning microscope. Following analysis, Images were adjusted in FIJI (Schindelin et al., 2012) and Adobe Photoshop CS6 for brightness and contrast but otherwise remained unaltered.

**Injury volume analysis**

The area of a visible cortical cavity and immunostained glial scar was measured in 20µm thick serial coronal sections at 5X magnification at 200 µm intervals using FIJI (Schindelin et al., 2012). Only cortical gliosis surrounding the injury was considered. Measurements were taken anteriorly from the first instance of scar moving posteriorly through to when the scar disappears. Values were used to estimate the total volume of the injury by averaging the area measured in each section and multiplying that value by the total length of the scar as calculated by the number of consecutive sections in which the scar was present.

**Gliosis measurement and analysis**

A representative set of serial coronal sections (20µm thick) from tissues immunostained for GFAP+ expression were visualized at 5X magnification at 200 µm intervals using FIJI (Schindelin et al., 2012). The total area of GFAP+ expression was measured in each section. A total gliosis volume was calculated for a subset of brains by averaging the area in all sections over the length of the identified scar. Once all sections were measured, the 20µm section exhibiting the maximal GFAP+ area was identified in each brain. After confirming that a strong, positive correlation exists between the maximal GFAP+ area and the total gliosis volume using regression analysis, we used the maximal GFAP+ area found in a 20µm section in each brain as a measure of gliosis.

**Cellular characterization and quantification**

For *in vitro* analysis, cells cultured for 5–7 days were fixed by treatment with 4% PFA for 20 minutes at room temperature followed by three 5-minute washes with 0.1M PBS. Three cell culture wells stained for each specific antibody (Oct4, Sox2, Ki67, βiii-Tubulin, Olig2, GFAP; **Table 1**) were counted within the field of view in five areas within each well at 20x magnification. Cells were counted by hand and the percentage of each cell type were calculated
as the number of Hoechst labeled cells expressing the antibody over the total number of Hoechst stained cells in each field of view.

For in vivo analysis, coronal sections 20 µm thick at 200 µm intervals were immunostained using their respective antibodies (Table 1). Transplanted drNPCs were identified using HuNu or STEM121 primary antibody. Total number HuNu+/Hoescht+ cells were counted manually. Total survival numbers were calculated by extrapolating the average number of surviving drNPCs per section over the total number of sections that the drNPCs were visible in throughout the cortex. The number of sections analyzed per brain ranged from 15–20 sections, spanning an approximate distance of 3–4 mm. To determine survival percentage, the absolute number of surviving drNPCs over total transplanted drNPCs was calculated. To analyze proliferation, the numbers of Ki67+/HuNu+ cells were counted in the same representative sections and calculated as a percent of all HuNu+ cells.

To characterize cell differentiation in vivo post-transplantation after stroke, coronal sections of brains that received drNPC transplants were stained with GFAP (astrocyte), NeuN (neuron), and Olig2 (oligodendrocyte) primary antibodies, followed by appropriate secondary antibodies. The stained sections were visualized using an AxioVision Zeiss UV microscope or Olympus FV1000 confocal point-scanning microscope.

**Polymerase chain reaction**

Cultured drNPCs were collected into Buffer RL (Norgen Biotek) with β-mercapthenol and then processed according to the manufacturer’s directions using Total RNA Purification Kit (Norgen Biotek – Cat#17200).

For RT-PCR, the cDNA synthesis was carried out with SuperScript® First-Strand Synthesis System for RT-PCR (ThermoFisher – Cat# 11904018). RT-PCR was carried out on GeneAmp PCR System 9700. Cycling conditions consisted of polymerase activation and DNA denaturation (3 min at 98°C), followed by 35 cycles of 10 s at 95°C and 30 s at 60°C. Primer sequences used are listed in Table 3.

For RT-qPCR, the cDNA synthesis was carried out with iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad – Cat# 1725034). RT-qPCR reactions were prepared according to the manufacturer’s directions using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad – Cat# 172-5270). RT-qPCR was carried out on Bio-Rad CFX384 Touch Real-Time PCR System.
Cycling conditions consisted of polymerase activation and DNA denaturation (3 min at 98°C), followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. All reactions were concluded by incubation at 65°C and increasing the temperature (at 0.5°C increments) to 95°C for melting-curve analysis. Bio-Rad SYBR Green Assays: CD34 (qHsaCID0007456), PRPH (qHsaCID0023851), SNAI1 (qHsaCED0057267), MPZ (qHsaCED0021475), GAPDH (qHsaCED0038674), and ACTB (qHsaCED0036269).

**Behavioural tests**

Mice were tested on a battery of sensorimotor assays (Cylinder Test, Foot Fault Test, Tape Test) at baseline and 4 days post-stroke (day of sacrifice) as a means measure tissue damage and functional deficits and evaluate the ET-1 model of stroke in immunocompromised SCID/Beige mice. In order to ensure long-lasting functional deficits, we conducted the foot fault test and cylinder test prior to stroke, 8 days following stroke, and at 32 days following stroke.

Mice used to evaluate the therapeutic potential for drNPC transplants were tested for behavioural sensorimotor function and deficits using the foot fault test prior to stroke, following stroke, and at various times following transplant to evaluate changes in performance. Specifically, mice were tested 3 days prior to stroke, 3 days post-stroke (1 day prior to transplant), and 8, 18, and 32 days post stroke (Figure 4C).

All animals were singly housed for the duration of behavioural testing schedules and were tested individually at consistent times throughout the day for the duration of the experiment. All behavioural tests were recorded with a digital camera (SX 60 HS, Canon) and viewed on VLC Media Player (Version 2.2.1, VideoLAN Organizarion) or Avidemux (Version 2.6.8, Open-source). All videos were scored by a blinded observer.

**Tape Test**

The Tape Test measures fine sensorimotor functionality by assessing sensory and motor attributes in forepaw use. To do so, a 0.3 cm X 0.4 cm piece of tape is applied to the central hairless portion of the forepaw and the time it takes each animal to contact it with the mouth and remove it is measured. To carry out this test, mice were exposed to the tape for 2 days (pre-training), followed by 5 days of training until performance reached plateau, and then tested for 2 days for each testing time point. All tests were done once per individual paw per day, for a total
of two daily trials. A total of 2 measures were taken: 1) elapsed time from beginning of trial to tape contact, and 2) elapsed time from contact to tape removal.

**Cylinder Test**

The Cylinder Test is used to measure forepaw preference in normal mouse rearing behavior. To conduct this test, mice were placed inside an elevated plexiglass cylinder for 4 minutes starting after the first paw contact with the wall of the cylinder. Paw “touches” (contact with the cylinder wall) were counted per paw, and a % paw preference score was calculated using the following formula: 

\[
\frac{\text{Contralateral Paw Touches} - \text{Ipsilateral Paw Touches}}{\text{Total Paw Touches}} \times 100
\]

Importantly, simultaneous touches with both paws were counted as “both” and only included in the “Total Paw Touches” calculation. Only animals with at least 20 paw touches were included in our analysis.

**Foot Fault Test**

The Foot Fault Test measures gross motor functions such as coordination and balance, as well as fine motor function like reaching and stepping. To perform the Foot Fault test, mice were placed on a 1 cm x 1 cm elevated grid and allowed to explore for 3 minutes. The total number of steps and slips (faults) per forepaw were counted. We then calculated the % Fault Slippage using the following equation:

\[
\frac{\text{(contralateral faults)-(ipsilateral faults)}}{\text{total steps}} \times 100
\]

to establish functional deficits or the % Fault Difference using the following equation:

\[
\left(\frac{\text{Contralateral faults}}{\text{Contralateral steps}} - \frac{\text{ipsilateral faults}}{\text{ipsilateral steps}}\right) \times 100
\]

to assess long term deficits and functional recovery. Using % Fault Difference removes the assumption that mice take equal number of steps between each paw, yet maintains the stability of comparing the injured paw to the uninjured paw.

**Statistical analysis**

All data is reported as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics (International Business Machines Corp., Armonk, NY). We performed a one-way analysis of variance (ANOVA) to evaluate differentiation profile of drNPCs in vitro. In establishing the
functional deficits of SCID/Beige mice, for the short term (4 days post-stroke) foot fault we used a repeated measures two-way ANOVA, for the long-term deficits in the foot fault we used a repeated measures one-way (time) ANOVA, to confirm unilateral deficits in the foot fault we conducted a repeated measures two-way ANOVA, for the tape test we used a repeated measures two-way repeated measures ANOVA, and for the cylinder test we ran a repeated measures two-way ANOVA. All test to establish deficits in SCID/beige mice were conducted with a Sidak post-hoc test. To analyze drNPC survival and proliferation within the lesioned tissue, we conducted a two-way ANOVA (day post-stroke, vehicle) to compare between vehicles at each time point and between survival at each time point for each vehicle. A Sidak post-hoc test was then conducted. The p-values reported represent adjusted p-values accounting for multiple comparisons using the post-hoc test. To analyze the functional recovery, we first conducted a repeated measures two-way ANOVA (compared to baseline) with a Sidak post-hoc test. We then conducted a repeated measures generalized linear model and identified a within-subjects cell × time interaction, which we further explored using a repeated measured two-way ANOVA with a Sidak post-hoc test. Analysis of lesion volumes was conducted with an unpaired T-test when comparing between day 4 and day 32 stroke only (untreated) brains, an ordinary one-way ANOVA with a Tukey post-hoc test when comparing all treated and untreated groups at day 32, and a two-way ANOVA with a Sidak post-hoc test when comparing between treatment groups (vehicles and transplants). The p-values reported represent adjusted p-values accounting for multiple comparisons using the post-hoc test. All correlations were calculated by conducting a bivariate Pearson’s correlation (two-tailed) between groups of interest.
Table 1: Primary antibodies used in this study

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Table 2: Secondary antibodies used in this study

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5. Results

5.1 drNPCs are a neurally committed population of cells

We characterized the drNPCs at the time of transplant using immunocytochemistry (ICC) and polymerase chain reaction (PCR). Cells were cultured to ~80% confluence within 5 – 7 days in vitro. Sister cultures were prepared for in vitro analysis and transplantation. For ICC, Oct4 expression was used as a measure of pluripotency; Sox2 to identify neural stem cells; Ki67 to assess proliferation; GFAP to identify astrocytes; βIII-Tubulin to identify neurons; and Olig2 to identify oligodendrocytes. We found that drNPCs do not express Oct4, but do express Sox2 and Ki67, and can differentiate to express GFAP, Olig2, or βIII-tubulin (Figure 5Ai-ii). The relative percentage of differentiated cells expressing markers for neurons (βIII tubulin; 27% ± 2.7), astrocytes (GFAP; 31% ± 1.4) and oligodendrocytes (Olig2; 24% ± 2.3) was not significantly different at the time of transplant occurring after 5 days in culture (One-way ANOVA, p=0.20; n=3 wells/group in 1 plate) (Figure 5B). Similarly, PCR confirmed that passaged drNPCs are neurally committed, expressing the neural lineage markers Nestin, Sox2, Ascl1, Pax6, MAP2, and CD133, and do not express the pluripotency markers Nanog and Oct4 (Figure 5C). By means of qPCR, we also confirmed that drNPCs do not express neural crest cell markers P0, peripherin, or the blood cell marker CD34, which is a marker expressed by the cells prior to reprogramming, since the batch of drNPCs we analyzed was originally reprogrammed from blood cells (data not shown). This data verifies that drNPCs did not maintain characteristics of cells prior to reprogramming. Collectively, these results confirm that drNPCs are neurally committed, and give rise to all three neural cell types.

When we analyzed the proliferation of drNPC sister cultures fixed at the time of transplantation, we found that 75.9% ± 0.1 of drNPCs are proliferative, indicating that drNPC proliferate in vitro.

5.2 ET-1 Stroke leads to sustained motor deficits in SCID/Beige mice

Transplantation of human derived precursors requires the use of an immunocompromised mouse strain (SCID/Beige mice) to avoid host immuno-rejection. For our study, we used the ET-1 model of cortical stroke at coordinates that target the forepaw region of the sensorimotor cortex and leads to sensorimotor behavioural deficits on the foot fault task. This has been demonstrated in immunocompetent C567BL/6 mice (Ballios et al., 2015; Sachewsky et al., 2014), and also verified using an immunocompromised NOD-SCID mouse PVD model (Erlandsson et al., 2011).
We performed the ET-1 cortical stroke and examined the functional impairments to confirm that SCID/Beige mice exhibited significant sensorimotor deficits that were maintained up to one month post-stroke. We tested baseline function of animals prior to stroke, and then at short (4 or 8 days) and long (32 days) time points following stroke. On the foot fault task, SCID/Beige mice that received an ET-1 induced stroke exhibited functional deficits as early as 4 days post-stroke (Figure 6A), at which time they were sacrificed for tissue analysis. The deficits were maintained at 8 days post stroke and persisted up to 32 days post-stroke, the longest time examined (Figure 6B). We confirmed that the deficit was due to contralateral paw impairments (contralateral to the stroke) by analyzing each limb separately and showing that the contralateral limb performed poorly compared to the ipsilateral limb following stroke (Figure 6C). Hence, the ET-1 cortical stroke model in SCID/Beige mice permitted us to examine functional recovery in our transplantation paradigm.

To investigate whether SCID/Beige mice exhibited deficits in other behavioural tasks and increase our battery of functional tests, SCID/Beige mice were also tested in the Adhesive Removal Test and the Cylinder Test. We have shown that ET-1 cortical stroke results in sensorimotor deficits in the Tape Test in immunocompetent C57BL/6 mice (Nusrat et al., 2015). However, no deficits in the Tape Test were observed in the immunocompromised SCID/Beige mice (Figure 6D,E), making it an inadequate test for our study. In the cylinder test which tests for asymmetry of paw use, we observed a significant deficit at early times post stroke and spontaneous recovery by 32 days compared to baseline and a naïve group (Figure 6F). This indicates that the cylinder test may be useful in identifying rate of recovery but may ultimately not be the optimal test for functional recovery, as stroke-only mice spontaneously recover to baseline and naïve levels by 32 days post-stroke. The inability to detect a sustained deficit in the cylinder test lead us to consider this behavioural test inappropriate for our recovery studies.

### 5.3 *drNPCs survive in tissue up to a month following transplant*

To determine whether drNPCs were able to promote functional recovery and tissue repair following Et-1 stroke, SCID/Beige mice were transplanted with cells at 4 days post-stroke, a time chosen based on previous success elucidating functional recovery using mouse NPCs (Ballios et al., 2015). We examined the number of drNPCs in the ipsilesional stroke-injured cortex at 8 and 32 days post-stroke (4 and 28 days post-transplantation) using
immunohistochemistry for human nuclei (HuNu) in a subset of brains (Figure 7A). The total number of drNPCs at day 8 post stroke was 12,780 ± 2,963 (n=4) when delivered in aCSF and 21,633 ± 9,880 (n=4) when delivered with HAMC (Figure 7B) representing 13% ± 3.0 and 22% ± 9.9 of the original transplanted population (100,000 cells) in aCSF and HAMC, respectively. There was no significant difference in the number of drNPCs in the ipsilesional cortex at 8 days post-stroke between the vehicles (p=0.34). By 32 days post stroke, the number of cells was reduced to 4,961 ± 1,266 (5.0% ± 1.3 survival) for drNPCs transplanted in aCSF (n=7) and 5,130 ± 1,815 (5.1% ± 1.8 survival) for those transplanted with HAMC (n=7). Similar to day 8 post-stroke, there was no significant difference (p=0.99) in the number of drNPCs following transplant between the two vehicles (Figure 7B). Although there was a decrease in the number of cells from day 8 to day 32 post-stroke with both transplant vehicles, only the cells transplanted in HAMC had a significant decrease in survival (aCSF, p=0.34; HAMC, p<0.05). Hence, while cell death may be delayed in the drNPC+HAMC group, drNPCs survive for 32 days irrespective of the transplant vehicle (aCSF or HAMC).

Next, we examined proliferation of the transplanted drNPCs by immunohistochemistry using Ki67. The number of Ki67+/HuNu+ cells as a percent of all HuNu+ cells in the ipsilesional tissue revealed that at 8 days post-stroke (4 days post-transplant) 8.5% ± 1.5 (aCSF, n=4) and 11.0% ± 2.0 (HAMC, n=4) of drNPCs were Ki67+, while at 32 days post-stroke (28 days post-transplant) Ki67− was expressed in 6.8% ± 1.3 (aCSF, n=6) and 8.2% ± 1.0 (HAMC, n=3) of HuNu+ cells within the ipsilesional tissue (Figure 7C). There was no significant difference between the two vehicles at 8 or 32 days post-stroke (day 8, p=0.49; day 32, p=0.79).

Furthermore, at 32 days post-stroke (28 days post-transplantation), the percent of Ki67− drNPCs was not significantly reduced compared to 8 days post-stroke irrespective of the vehicles (aCSF, p=0.66; HAMC, p=0.46).

Given the observation that >75% of the drNPCs are proliferating in culture when they are harvested for transplantation, these data may suggest that drNPCs decrease in proliferation once transplanted in vivo following stroke or that proliferative cells died in the transplantation process.

5.4 Transplanted drNPCs promote functional recovery post-stroke

To evaluate functional recovery, mice were tested in the foot fault task at 3 days prior to stroke to establish a baseline measure, and at 3, 8, 18, and 32 days post-stroke. We confirmed
that naïve, uninjured animals (n=8) do not experience variability in performance across testing days (data not shown). To assess the therapeutic effects of drNPC transplants, only stroke-injured mice that had significant motor impairments by 8 days post-stroke relative to their baseline controls were included in our analysis (aCSF = 11/12; HAMC = 11/13; drNPCs+aCSF = 10/13; drNPCs+HAMC = 14/15).

We observed a significant functional deficit at all times post-stroke in mice that received vehicle only injections (aCSF or HAMC). Mice that received drNPC transplants on day 4 post-stroke, in either HAMC or aCSF, displayed functional deficits up to day 18 post-stroke (14 days post-transplant) but recovered to baseline performance at 32 days post-stroke (Figure 8A). Our analysis revealed a cell × time interaction whereby a significant difference in performance between mice that received vehicle only or drNPCs+vehicle was seen at day 18 and day 32 post-stroke (Figure 8B). Furthermore, we found a correlation between functional performance on day 32 post-stroke and the presence of drNPCs in the cortex (p=0.03), but not their absolute number (p=0.27). Importantly, the observed functional recovery in mice that received drNPC transplants occurs regardless of sex (Figure 8C). These findings reveal that drNPCs are sufficient for recovery and can promote improvement regardless of the delivery vehicle or sex of recipient.

5.5 Transplanted drNPCs differentiate in vivo

We analyzed the in vivo differentiation profile of drNPCs transplanted in aCSF and HAMC at 8 and 32 days post-stroke (4 and 28 days post-transplant). The drNPCs were first identified within the cortical tissue using either HuNu+ or STEM121+ antibodies. At all times, cells were present in the stroke injured cortex, irrespective of the vehicle (Figure 9). We performed immunohistochemistry for GFAP (astrocytes), NeuN (neurons) and Olig2 (oligodendrocytes) and confirmed that some HuNu+ or STEM121+ stained cells within the cortex co-localized with these lineage markers. We found drNPCs expressed GFAP at 8 and 32 days post stroke. We did not observe human cells expressing NeuN+ or Oligo2+ cells at 8 days post stroke, however by day 32, a few transplanted cells expressed NeuN or Olig2. Similar to what has been previously reported with mouse NPCs (Ballios et al., 2015), this qualitative analysis suggests that transplanted cells primarily differentiate into GFAP expressing astrocytes in the stroke injured brain.
5.6 Stroke lesions are not affected by cell or vehicle

To determine the extent of the ischemic lesion following ET-1 stroke, we examined the lesion volume as measured by the extent of the glial scar and physical cavity at 4 and 32 days following ET-1 stroke. The glial scar was assessed with immunohistochemistry using GFAP to stain reactive astrocytes. Visible lesions were found in the stroke injured cortex, but not in contralateral hemispheres, at both 4 and 32 days post-stroke (Figure 10A). We found that the lesion volume was significantly reduced (p=0.01) between day 4 (1.55 mm$^3$ ± 0.28 mm$^3$; n=5) and day 32 (0.43 mm$^3$ ± 0.16 mm$^3$; n=4) post-ET-1 stroke (Figure 10C). There was no correlation between functional performance and tissue damage at day 32 post-stroke when we explored the relationship between stroke lesion volume and functional outcomes in these stroke-injured mice ($r =0.23$, $n=3$, $p=0.85$). This suggests that while there was no improvement in foot fault behaviour, there was a significant decrease in the size of the lesion over time in untreated mice.

Next, we compared the extent of tissue damage in treated (vehicles or vehicles+drNPCs) brains 32 days post-stroke ($n \geq 5$ per group). All mice had cortical lesions (Figure 10B). Interestingly, at 32 days post-stroke there was no significant difference in lesion volumes between any of the treated groups or mice that received stroke only without any treatment (Figure 10C). No correlation was seen between functional performance on the foot fault at 32 days post-stroke and lesion volumes ($r = -0.05$, $n=23$, $p=0.82$). These results suggest that drNPCs and/or vehicles do not affect the extent of lesion as measured by glial scarring and physical damage. A Pearson’s correlation was run to validate the relationship between total gliosis volume and the maximal GFAP$^+$ area in a 20 µm section per brain. We found a strong, positive correlation between the gliosis volume and maximal GFAP$^+$ area ($r=0.868$, $n=25$, $p<0.001$). Accordingly, we used the maximal GFAP$^+$ area as a measure of gliosis. Mice that received drNPCs in aCSF exhibited significantly increased GFAP$^+$ expression compared to mice that received aCSF alone ($p<0.05$). This was not observed in mice that received cells in HAMC compared to HAMC alone ($p=0.93$). There was no significant difference between the vehicle type when comparing mice that received cells ($p=0.11$) or vehicle injections alone ($p=0.90$). This suggests that aCSF is more permissive to drNPC-dependent mechanisms that can increase GFAP$^+$ expression; either greater drNPC differentiation into astrocytes or enhanced induction of host astrogliosis. Hence, drNPC transplants are sufficient for promoting functional recovery.
following stroke, without impacting the lesion volume post-stroke, and with an effect on gliosis that depends on transplant vehicle.
6. Discussion
Herein, we have shown that transplanting drNPCs is a promising approach to treating sub-acute ischemic stroke. Although cell transplants or vehicle did not affect the extent of visible tissue damage, transplanted cells survived up to a month within the injured tissue and promoted functional recovery, regardless of what vehicle they were transplanted in. The proliferation of drNPCs decreased once cells were injected into the stroke-injured brain. A subset of drNPCs within the cortical tissue preferentially differentiated into astrocytes, with fewer drNPCs giving rise to neurons and oligodendrocytes. These results indicate that drNPCs can survive following transplant and are able to provide therapeutic benefits that result in functional recovery in the absence of neuronal differentiation.

6.1 The ET-1 stroke paradigm in SCID/Beige mice
Since we were working with human cells, we developed a novel ET-1 stroke paradigm in the immunocompromised SCID/Beige mouse strain that resulted in persistent long-term functional deficits following stroke. Through our battery of behavioural tests (foot fault, tape test, and cylinder test), we found that the SCID/Beige mice sustained deficits up to a month following ET-1 stroke only in the foot fault task. One possibility that may explain why we did not observe long-term deficits post-stroke in the tape test and cylinder test is the fact that the immune system is compromised in these animals. Indeed, there is increasing evidence to suggest that the immune system plays a complex role in the prognosis of stroke (Iadecola and Anrather, 2011; Rayasam et al., 2017). In our lab, we have shown that immunodeficient NOD/SCID mice spontaneously recover following PVD stroke compared to immunocompetent NOD/LTJ mice (Erlandsson et al., 2011). Moreover, the administration of Cyclosporin A, an immunosuppressant, has also been shown to promote functional recovery (Abeysinghe et al., 2015; Sachewsky et al., 2014), suggesting that the immune system may play a detrimental pro-inflammatory role in the response to stroke. It also so possible that the behavioural tests we used may not be sensitive enough to detect deficits in our mouse model. For example, the cylinder test may not optimal for use in mouse models of small cortical ischemia, but might be more relevant in rats or in mice with more severe injuries (Balkaya et al., 2013). Furthermore, since the cylinder test assesses paw preference and the tape test assesses fine sensorimotor skills, our paradigm may have only affected gross motor skills such as coordination and balance, which would explain the sustained
deficits found only in the foot fault test. It is also possible that the organization of the brain could vary slightly between mouse strains, as we had previously documented functional deficits in the tape test when we performed the same paradigm in immunocompetent C57BL/6 mice (Nusrat, 2015; Nusrat et al., 2015). Since the only difference between the two experiments was the mouse strain, our data suggests that the reason for our observations are likely due to the differences between mouse strains, whether it be the immune system or brain organization, and not the behavioural assays themselves. Future studies should consider implementing a larger battery of behavioral tests to allow for a deeper investigation of functional deficits and recovery. Importantly, our ability to identify a persistent functional deficit in at least one behavioural test allowed us to evaluate functional recovery following drNPC transplantation.

The implications of using an immunocompromised SCID/Beige mouse for our stroke model go beyond differences in behavioural deficits. We chose this mouse strain, as it is the gold standard for translational human xenograft studies in mice (Food and Drug Administration (FDA), 2008). The SCID/Beige mouse lacks B and T cells, and has dysfunctional natural killer (NK) cells; this means that it has severely immunocompromised adaptive immunity but components of innate immunity remain unchanged. Thus, if any of the affected cells play a key role in the progression of stroke injury, then this may indicate that a SCID/Beige stroke may be less severe than that observed in immunocompetent counterparts. There is substantial evidence to suggest that lymphocytes, such as B –cells, and especially T-cells, may be involved in the inflammatory response following stroke (Hum et al., 2007; Jin et al., 2010b, 2013; Rayasam et al., 2017). Immunocompromised mice lacking B and T cells appear to be protected from ischemic injury, as they exhibit reduced lesion sizes and inflammation compared to immunocompetent controls (Hum et al., 2007). Assuming that our SCID/Beige mice would also exhibit this resistance to ischemia, we had originally doubled the concentration dose of ET-1 from 400 picomolar to 800 picomolar and further, implemented our battery of behavioural tests to identify if any would be able to detect long term deficits. It is possible that if we had increased our ET-1 dose further, we may have found a greater severity of tissue damage and degree of functional deficits in the foot fault or other behavioural tasks.
6.2 drNPC transplantation in SCID/Beige mice

The fact that SCID/Beige mice lack adaptive immunity also raises the question of whether transplanted cells would survive better in SCID/Beige mice compared to immunocompetent animals, as they would not be attacked by the lymphocytes. This poses a challenge to the translatability of our paradigm as we sought out to test human cells in an animal model. However, transplanted cells are still exposed to microglia and infiltrating macrophages which are present in SCID/Beige mice, activated following stroke, and have been shown to influence graft survival (Buhnemann et al., 2006; Hoornaert et al., 2017). In any case, since drNPCs can be reprogrammed directly from a patient, autologous transplantation would avoid the need for immunosuppression and is not expected to elicit an immune response. Regardless, future tests should investigate whether drNPCs reprogrammed from an animal can be transplanted directly back into that same animal following stroke to elucidate functional recovery without the need for immunosuppression.

Based on previous success from our group transplanting mouse NPCs using an ET-1 model of stroke (Ballios et al., 2015), we injected cells directly into the ischemic cavity 4 days after stroke with either HAMC or aCSF. In the study using mouse NPCs, we showed that cells transplanted in HAMC survive better than those transplanted in aCSF immediately after transplantation and show a trend towards increased survival up to a week post-transplant. Surprisingly, in our study using human drNPCs, we found no differences in cell survival between drNPCs transplanted in HAMC or aCSF at 4 or 28 days following stroke. We also found that in this study, we had greater overall cell survival than previous work; by day 4 post-stroke, we found the percent of surviving cells in either vehicle to be more than twice as much as the mouse NPCs transplanted in C57BL/6 mice that were analyzed immediately after transplantation and even more than those found 7 days later (Ballios et al., 2015). Although we looked at different time points, our results could indicate that either drNPCs can survive regardless of the vehicle transplant or that SCID/Beige mice are more permissive to xenograft survival. Furthermore, HAMC has been shown to have immunomodulatory effects (Austin et al., 2012; Cooper et al., 2008; Gupta et al., 2006; Wang et al., 2012b). Thus, if HAMC had an immunomodulatory effect in C57BL/6 mice that allowed cells to survive, we would not expect to see differences between the two vehicle transplants in SCID/Beige mice.
Although reports indicate that HAMC has pro-survival properties (Ballios et al., 2010, 2015; Caicco et al., 2013; Hsieh et al., 2010), we did not find any differences between the HAMC hydrogel and aCSF in terms of cell survival. Improving cell survival can be achieved through a variety of methods such as preconditioning cells to hypoxic environments, modifying the cells prior to transplant, co-transplanting with other cells that secrete growth factors, delivering cells within biomaterials (hydrogels, scaffolds, nanotubes, and matrices), and/or administering drugs and pro-survival factors to provide trophic support (Cooke et al., 2010; Dadwal et al., 2015; Doeppner et al., 2012; Donaghue et al., 2014; Jin et al., 2010a; Moon et al., 2012; Qiao et al., 2014; Sachewsky et al., 2014; Sart et al., 2014; Wang et al., 2012a, 2012b; Yu et al., 2013). Alternatively, it is also possible that increasing cell survival will not necessarily lead to further benefits. Of note, just because cells are surviving does not mean they are integrating into the tissue, which is something that should also be considered. Increasing cell integration into the cortical tissue may result in better outcomes or more rapid recovery. This could be also achieved with the use of nanoscaffolds or guided micropatterned implants that can effectively provide a “foothold” for cells and aid in migration and integration (Moon et al., 2012; Vaysse et al., 2015). This approach may induce greater regeneration and functional recovery than we observed in the current study. However, there is also substantial evidence that non-neural cells and cells delivered via the vasculature exhibit little to no engraftment, yet can lead to functional recovery (Banerjee et al., 2011; Chen et al., 2016; Ikegame et al., 2014). Thus, it is not out of the realm of possibility to think that NPCs may also be able to act through similar mechanisms and need not to engraft to provide benefits.

It is also important to note that we did not find any tumor formation in any of our mice. Although our timeline may have been too short for the development of tumors, previous work with drNPCs has shown no sign of tumorigenesis or teratoma formation (Azimi, 2016; Khazaei, 2016, 2017). This supports the claim that drNPCs are safe for transplantation.

### 6.3 Role of drNPCs in promoting recovery following stroke

When we investigated the differentiation of transplanted drNPCs in vivo we found that a majority of cells are differentiating into astrocytes (GFAP-positive cells), with few cells giving rise to neurons (NeuN-positive cells) and oligodendrocytes (Olig2-positive cells). This differentiation profile differed from that of cells that were cultured in vitro where we found that
drNPCs did not preferentially differentiate into a specific cell type. However, since the cells were transplanted directly into the lesion cavity; an environment that would promote differentiation towards an astrocytic lineage (Faiz et al., 2015), this was not surprising to us. Following stroke, a cascade of immune events occurs that eventually lead to the formation of the glial scar. It would not be surprising if drNPCs transplanted into the lesion cavity receive the same signals and thus participate in the scarring process. Previous work in our lab has shown that endogenous NPCs migrate from the SVZ towards the ischemic lesion, where they differentiate and give rise to reactive astrocytes (Faiz et al., 2015). Similarly, when NPCs are transplanted following stroke, they primarily gave rise to GFAP-positive astrocytes (Ballios et al., 2015; Chu et al., 2004; Tang et al., 2014), although they have also been shown to differentiate into neurons and oligodendrocytes (Buhnemann et al., 2006; Hayashi et al., 2006; Ishibashi et al., 2004; Kelly et al., 2004) or remain undifferentiated (Bacigaluppi et al., 2009, 2016). Interestingly, GFAP-expressing cells have been implicated in functional recovery, matching our observations in this study (Becerra-Calixto and Cardona-Gómez, 2017; Chu et al., 2004). These findings suggest that astrocytes, which are recognized to comprise a number of different subpopulations (Anderson et al., 2014; Chaboub and Deneen, 2012; John Lin et al., 2017; Khakh and Sofroniew, 2015; Liddelow and Barres, 2017; Oberheim et al., 2012; Rusnakova et al., 2013; Zamanian et al., 2012; Zhang and Barres, 2010), may play a role in functional recovery.

The goal of this study was to investigate whether drNPC transplants are efficacious in promoting post-stroke recovery. We found that transplanting drNPCs promotes recovery following stroke, however, the mechanism by which this occurs is still unknown. Herein we only investigated the extent of tissue damage, the presence of cells a month post-transplant, and their differentiation in vivo. Since we found no correlation between the extent of damage and behavioural performance, our results suggest that these two measures may not be related; one does not need to happen for the other to occur. This discrepancy is consistent with what has commonly been observed in other studies (DeVries et al., 2001). Furthermore, we only saw ~5% of transplanted cells survive up to a month following transplant, with many them differentiating into astrocytes. This may indicate that only a few cells are needed for functional recovery or that the drNPCs may be acting through other mechanisms and may not be participating in tissue sparing, neural integration, and tissue regeneration. Indeed, there is increasing evidence suggesting that transplanted cells can promote recovery through other mechanisms such as
providing trophic support, promoting plasticity, inducing angiogenesis, immunomodulation, and even activating endogenous cells to proliferate and migrate to the site of lesion (Andres et al., 2011; Bacigaluppi et al., 2016; Baraniak and McDevitt, 2010; Doepner et al., 2012; Drago et al., 2013; Hermann et al., 2014; Horie et al., 2011; Jiang et al., 2005, 2006; Mine et al., 2013; Minnerup et al., 2011; Oki et al., 2012; Satani and Savitz, 2016; Zhang et al., 2011). However, it is still difficult to completely disregard the notion that few cells may be integrating into the tissue and providing therapeutic benefits, as we didn’t test for neural activity using a presynaptic marker, electrophysiology, and/or calcium imaging to investigate whether the surviving and differentiated drNPCs are functionally integrating into the tissue. In any case, it is quite possible that the cells may be elucidating their therapeutic effects through more than one of these mechanisms.

Through our experiments, we found that cells can survive in tissue up to a month post-transplant and that their presence is correlated with improved functional outcomes. Yet, there is still the possibility that the survival of transplanted cells is not necessary for functional recovery to occur. To address this, future work should examine whether cells must survive and integrate into the tissue to promote and sustain functional recovery. One way to do this would be with an ablation paradigm, in which transplanted cells are ablated once animals recover. If the performance of animals remains consistent, it is possible that it is not necessary for cells to survive and that they may only be promoting recovery through the release of growth factors and anti-inflammatory signals in the early stages following transplants. Conversely, if the functional deficits return following ablation, it may indicate that drNPC transplant survival is not only sufficient, but necessary for recovery.

Interestingly, we found that we could elicit functional recovery without affecting the damaged tissue; there was no difference in the total lesion size between groups receiving vehicle or drNPC transplants even when compared to untreated mice. Though one might hypothesize the opposite to be true, there is evidence to suggest that these two outcomes may not be related, as others have also reported functional recovery without any effects on the brain tissue (Smith et al., 2012). One possible explanation for these results is neuroplasticity; the brain’s ability to change and reorganize over time. These changes can be achieved through tissue remodeling, cortical remapping, axonal sprouting, and the development of new synaptic junctions (Andres et al., 2011; Murphy and Corbett, 2009). It is possible that visible physical changes to the lesion are not
necessary to elicit a beneficial functional response. Alternatively, we acknowledge that our measure of lesion included the physical damage in conjunction with glial scarring. Future studies should attempt to analyze the extent of the lesion with more than one outcome measurement that can account for cell death specifically.

6.4 Tissue response to stroke

Although we didn’t find any changes in the extent of the lesion as a result of drNPC transplants at 32 days post-stroke, we did see significantly reduced lesion volumes at 32 days post-stroke compared to day 4 post-stroke. This is similar to what has been previously reported in that the extent of tissue damage may peak at short time points post-stroke and then gradually decline (Li et al., 2014). Another possibility that may explain our finding is that we used GFAP to identify the extent of the lesion inclusive of the scar, and thus, at day 4 post-stroke may have also identified migrating NPCs in active gliosis (Faiz et al., 2015). By doing this, we may have extended the area measurements in our analysis because it is possible that we included migrating cells that may not have been part of the lesion or scar in our calculations.

Our findings that drNPC transplantation only increase GFAP expression when transplanted in aCSF suggest that HAMC may play a role in modulating drNPC-dependent astrocytosis. Additionally, our findings that HAMC does not affect GFAP expression agrees with prior research which suggests that HAMC or hyaluronan-based hydrogels alone do not affect GFAP expression compared to aCSF (Austin et al., 2012; Führmann et al., 2015). This further supports the idea that the interaction between drNPCs and transplant vehicle may be important in the observed results.

6.5 Future directions

In our study, animals were singly housed in cages without any enrichment starting from 3 days prior to stroke until sacrifice. This had a two-fold purpose; first, it prevented anxiety caused by aggressive cage-mate behaviour, and secondly, it mimicked the condition that many human patients experience, as there is an increase in the number of stroke patients living alone and feeling lonely (Steptoe et al., 2013). Nowadays, more and more people are experiencing loneliness, especially in the aging population. Thus, it is important to understand that social isolation has been shown to be detrimental for stroke recovery and this could have had a direct
impact on our studies. Even though we decreased the chances of observing benefits by using this housing paradigm, we were still able to observe functional recovery using cell transplantation. This strengthens the case for drNPCs as a potential therapeutic approach to treat stroke. Regardless, future studies should consider housing animals in a social environment to increase the chances of recovery and improve outcomes. Substantial evidence also exists to suggest that rehabilitation, exercise, and enriched housing may also play a key role in the recovery process (Chen et al., 2017; Livingston-Thomas et al., 2016; Mering and Jolkkonen, 2015; Venna and McCullough, 2015). Hence, by taking this into account and housing animals in such scenarios it may possible to induce even greater or faster benefits from drNPC transplants.

It is also important to recognize that we conducted our research using young adult mice of both sexes. Thus, although we accounted for sex in our study by incorporating both sexes, the fact that we used young adult mice is different from humans, in which the group that is most often affected by stroke is the elderly population. More than half of all strokes occur in people over 75 years of age; the average age for stroke onset in males is 69.8 years and 75.3 in females (Feigin et al., 2003). An interesting avenue to explore is whether drNPC transplants are also efficacious in an aged population.

Another consideration to make is the limitation of our transplant paradigm; we transplanted 100,000 drNPCs directly into the lesion cavity 4 days following stroke. Although we saw improved functional outcomes and cells surviving up to a month post-transplant, it is possible that our transplant parameters may still not have been optimal for drNPCs. It would be interesting to test the transplant success of drNPCs by changing a variety of factors, such as dosage, time of transplantation, transplant speed, transplant location, route of administration, and transplant vehicle, in order to identify the best parameters to transplant drNPCs. By optimizing transplant parameters, we may be able to improve outcomes of drNPC transplants.

Considering that multiple clinical trials have been carried out to identify the safety and efficacy of cell transplantation strategies following stroke. A large majority of these trials have focused on testing non-neural cell types, such as MSCs, BMSCs, and UCBCs (Bang, 2016; Honmou et al., 2011; Kurtzberg et al., 2016; Rosado-de-Castro et al., 2013; Taguchi et al., 2015). Several of these trials have confirmed the safety of the cells transplanted and reported benefits following transplantation, yet the most elusive challenge of neuro-regeneration still remains. Transplants using non-neural cells are unable to regenerate the tissue that is lost following stroke.
since these cells lack the ability to differentiate into neural cell types. Only a limited number of clinical studies have been performed using cells specific to the neural lineage (Kalladka et al., 2016; Kenmuir and Wechsler, 2017; Qiao et al., 2014; Savitz et al., 2005). Yet, these studies were limited by the use of allogenic cell sources making immune rejection a significant barrier to recovery. One study confirmed the immune safety of cells prior to transplant by using blood to test the human leukocyte antigen class I and II antibody response to cells in order to exclude patients that would develop adverse immune effects, thereby limiting the population that would be able to access this therapy (Kalladka et al., 2016). However, this model would be difficult to efficiently apply in the clinic, as each patient must be tested separately, and not all patients would qualify for transplantation. Other clinical studies using NPCs have reported adverse events as a result of the intervention (Qiao et al., 2014; Savitz et al., 2005). It is clear that there is still major knowledge gap that needs to be filled in order to safely translate NPC transplant therapies to the clinic. Since we have shown recovery of drNPC transplants in a sub-acute ET-1 model of sensorimotor stroke using immunocompromised SCID/Beige mice, it is important that prior to translating drNPC transplants to clinical trials, their therapeutic effects be tested in a chronic model of stroke, in different stroke models, using immunocompetent animals, in a model that results in cognitive deficits, and in non-human primates, directly reprogramming the primate’s own cells. Furthermore, it would be interesting to test the benefits of drNPC transplants in conjunction with other therapeutic approaches, such as metformin administration or rehabilitation (or a combination), since these also have the potential to promote recovery and may have synergistic effects. Clearly, drNPCs are beneficial in treating stroke, but it is also possible that we have yet to explore their full potential as a therapeutic approach.
7. Conclusion

We have shown the therapeutic efficacy of human drNPC transplants for subacute ischemic stroke using an immunocompromised mouse model of stroke. Our results indicate that drNPCs may be a viable source of cells for clinical application. Each of the outlined aims was addressed to provide evidence and support for our findings.

Aim 1: Characterize drNPCs at time of transplantation

Our in vitro characterization at time of transplantation revealed that drNPCs are committed to the neural lineage and do not preferentially differentiate to a specific cell type when cultured prior to transplantation.

Aim 2: Develop and characterize the ET-1 model of stroke in SCID/Beige mice

We successfully developed an ET-1 model of sensorimotor stroke in SCID/Beige mice. In our model, SCID/Beige mice exhibit functional deficits in the foot fault task that arise within 4 days post-stroke and persist up to a month post-stroke. Tissue analysis reveals that this stroke model results in observable tissue damage and gliosis, which can be detected at short and long time points following stroke.

Aim 3: Investigate the therapeutic relevance of drNPC transplants for stroke recovery

We transplanted drNPCs to investigate their potential as a therapeutic approach to stroke. Our results demonstrate that drNPCs survive up to one month within the transplanted tissue, promote functional recovery, and do not affect the lesion volume following ET-1 stroke in SCID/Beige mice. Our in vivo characterization data suggests that drNPCs mainly express GFAP, indicating that they preferentially differentiate into astrocytes following transplantation after stroke. We did not find any difference between the two delivery vehicles (aCSF or HAMC) in our analysis.
Figure 1: Directly reprogramming somatic cells to NPC's bypasses pluripotency

Somatic cells can be directly reprogrammed to drNPCs without the use of viral constructs via transient expression of Musashi-1 (Msi1), Neurogenin-2 (Ngn2), and Methyl-CpG Binding Domain Protein 2 (MBD2). This process bypasses a pluripotent stage and results in drNPCs that can give rise to neurons, astrocytes and oligodendrocytes.
Figure 2: Stroke results in a central ischemic core surrounded by a penumbra region
Following a stroke, the ischemic core, which makes up the center of the lesion, receives little to no blood flow and is characterized by rapid cell death (necrosis and apoptosis) that results in irreversible damage. The penumbra is the surrounding region of viable but damaged neurons that are supported by unaffected collateral arteries and can be rescued with reperfusion if blood flow remains limited and no reperfusion occurs, cells in this area may also undergo apoptotic cell death.
Figure 3: Time course of events following ischemic stroke in rodent models
The progression of stroke occurs over a span of overlapping phases that start immediately after a stroke and can span up to several months after. In rodents, the acute phase starts within minutes following stroke, when rapid cell death occurs that results in danger signals being released into the body. This is followed by the subacute phase, which starts around 48 hours following stroke and usually lasts up around 2 – 3 weeks. In the subacute phase, cell death continues to occur in conjunction to a major inflammatory response which eventually stabilizes and results in the chronic phase of stroke. Most neuroprotective therapies aim to modulate the inflammatory response in the subacute phase, while rehabilitative approaches aim to improve plasticity in the chronic phase.
Figure 4: Experimental paradigm to test the therapeutic efficacy of drNPCs
Therapeutic efficacy of drNPCs was assessed using a functional task and tissue analysis. All animals received an ET-1 induced stroke in the sensorimotor cortex. (A) Animals were randomly separated into one of four testing groups following stroke. (B) The drNPCs were cultured in monolayers for 5-7 days until they reached ~80% confluence. On the day of transplantation, cells were detached, suspended in either HAMC or aCSF, and transplanted into the injury site at 4 days following stroke. (C) Mice were tested for functional performance using the foot fault task at predetermined time points and sacrificed 32 days post-stroke, when tissue analysis was performed.
**Figure 5: drNPCs are a neurally committed population of cells**

(A) Immunocytochemistry reveals that drNPCs do not express the pluripotency marker Oct4 (Ai), express neural stem cell marker Sox2 (Aii), proliferate in vitro as indicated by Ki67+ (Aiii) and differentiate into neurons (Aiv), oligodendrocytes (Av), and astrocytes (Avi). (B) The numbers of differentiated neurons (βIII-tubulin), oligodendrocytes (Olig2), and astrocytes (GFAP) are not significantly different after 5 days in culture. (C) Polymerase chain reaction confirms that drNPCs express neural markers Nestin, Sox2, Ascl1, Pax6, MAP2, CD133, and do not express pluripotency markers Nanog and Oct4.
Figure 6: ET-1 stroke model leads to long term functional deficits in SCID/Beige mice

(A) SCID/Beige mice exhibited functional deficits in the foot fault test at day 4 post-stroke compared to naïve (unlesioned) controls. (B) Mice exhibited deficits at 8 days post-stroke that persisted up to 32 days post-stroke compared to baseline levels in the foot fault test. (C) Analyzing the performance of each paw separately shows that the contralateral paw was significantly impaired compared to the performance of the ipsilateral paw on the foot fault task. (D,E) No deficits were found in the tape test measuring the time to contact the tape (D) and the time to remove the tape (E) when SCID/Beige mice were tested 3 and 4 days prior to stroke and then again at 3 and 4 days post-stroke. (F) Mice exhibited functional deficits at 8 days post stroke that spontaneously recovered by 32 days post-stroke compared to baseline and naive levels in the cylinder test. * = p<0.05, R=Right; L=Left
**Figure 7: Transplanted drNPCs survive in the stroke injured cortex**

(A) Immunohistochemistry reveals the presence of drNPCs transplanted in either aCSF or HAMC within the stroke-injured cortex of SCID/Beige mice at 8 and 32 days post-stroke. (B) The number of HuNu^+^ drNPCs found within the stroke injured cortex at 8 or 32 days post-stroke was not significantly different between cells transplanted in aCSF or HAMC. (C) There was no significant difference between transplant vehicles in the percentage of Ki67^+^ drNPCs found in the ischemic lesion at 8 or 32 days post-stroke. n.s. = not significant.
Figure 8: Transplanted drNPCs promote functional recovery

All stroke-injured mice have a significant functional deficit following stroke. (A) Deficits persisted up to 32 days post-stroke in mice that received vehicle only injections, but not in drNPC transplanted mice, which recovered by 32 days post-stroke irrespective of the vehicle. (B) Mice receiving drNPCs, regardless of the vehicle, had significantly improved performance on the foot fault task at 18 and 32 days post stroke compared to those receiving vehicle injections. (C) There is no sex-dependent differences in recovery following cell transplantation, as both sexes display recovery when receiving drNPC transplants.

(A)

(a) = aCSF alone different from baseline; 
(b) = HAMC alone different from baseline; 
(c) = drNPCs+aCSF different from baseline; 
(d) = drNPCs+HAMC different from baseline, p<0.05

(B)

* = p<0.05

(C)

(w) = Male, Vehicle different from baseline; 
(x) = Female, Vehicle different from baseline; 
(y) = Male, drNPC different from baseline; 
(z) = Female, drNPCs different from baseline; 
(p) = p<0.05
Figure 9: drNPCs transplanted after stroke preferentially differentiate into astrocytes in vivo

Immunohistochemistry data reveals that transplanted drNPCs seem to express GFAP but not NeuN or Olig2 at 8 days post-stroke. Transplanted cells primarily differentiate into astrocytes (GFAP) and a small fraction of neurons (NeuN+) and oligodendrocytes (Olig2+) at 32 days post-stroke (white arrows). Scale bars = 50 μm.
Figure 10: Treatment affects gliosis but not total lesion volume

(A) Immunohistochemistry for GFAP+ reveals the presence of a glial scar and a physical cavity in the ipsilesional cortex but not the contralesional cortex at 4 and 32 days post stroke. (B) The lesion, as measured by the glial scar (GFAP) and physical damage was present in all treated brains at 32 days post-stroke. (C) The lesion volume is significantly reduced from 4 days post-stroke to 32 days post-stroke in stroke-only mice that received no treatment. The lesion volume is not significantly different between any of the treated groups or untreated group at 32 days post-stroke. (D) There is a strong, positive correlation between the total measured gliosis volume and the maximal GFAP+ area (in a 20 µm section) per brain. (E) The maximal GFAP+ area is significantly larger in mice receiving drNPCs within aCSF than aCSF alone. There was no significant difference between mice receiving drNPCS+HAMC or HAMC alone. No significant difference exits between vehicles in animals receiving cells or in animals receiving only vehicle injections. 

CC = Corpus Callosum; Cx = Cortex; Dashed line in images = Lesion area; Dashed lines in graph = 95% confidence band; n.s. = not significant; r = pearson’s coefficient; *= p<0.05; Scale bars = 500 µm.
9. Works Cited


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