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Transplantation of a novel xeno-free human neural stem cell population restores cognition in an immunodeficient rodent model of traumatic brain injury

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

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DEDICATION

To

My Mother, Grandmother, and Grandfather

I would like to dedicate this dissertation to my wonderful and amazing mother, grandmother, and grandfather. I want to thank you for raising me and instilling in me the values of hard work, dedication, and respectfulness of others and my profession. You have taught me so many valuable lessons, and you have supported me throughout all of my pursuits. You have dedicated part of your lives to raising me, and so it is the least I can do to dedicate this dissertation to you.

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ABSTRACT OF THE DISSERTATION

Transplantation of a novel xeno-free human neural stem cell population restores cognition in an immunodeficient rodent model of traumatic brain injury

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Doctor of Philosophy in Biomedical Sciences

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Traumatic brain injury (TBI) in humans can result in permanent tissue damage and has been linked to cognitive impairment that lasts years beyond the initial insult. Clinically effective treatment strategies have yet to be developed. Transplantation of human neural stem cells (hNSCs) has the potential to restore cognition lost due to injury, however, common methods for the generation of human embryonic stem cell (hESC) derived neural stem cells result in cells with potentially compromised safety profiles due to maintenance of cells in conditions containing non-human proteins (e.g. in bovine serum or on mouse fibroblast feeders) as well an increased risk of teratoma formation. Additionally, the vast majority of rodent TBI/hNSC studies to date have evaluated cognition only at early time points, typically less than 1 month post-injury and cell transplantation, and human cell engraftment and long-term survival in rodent models of TBI has been difficult to achieve due to host immunorejection of the transplanted human cells. To overcome these shortfalls, we have generated hNSCs in completely “Xeno-Free” (human only) culture conditions. Furthermore, we have enriched the hNSCs for the cell surface marker CD133 via magnetic sorting, which has led to an increase in the expansion rate and neuronal fate specification of

the hNSCs *in vitro*, and we have confirmed neural lineage specificity and a lack of teratoma formation upon sorted hNSC transplantation into the immunodeficient NOD-*scid* mouse brain. We then developed a novel TBI xenotransplantation model that utilizes immunodeficient athymic nude (ATN) rats as the host recipient for the post-TBI transplantation of human embryonic stem cell (hESC) derived NSCs and have furthermore evaluated cognition in these animals at long-term (≥2 months) time points. We report that immunodeficient ATN rats demonstrate hippocampal-dependent spatial memory deficits at 2-3 months post-TBI, confirming that ATN rats recapitulate some of the cognitive deficits found in immunosufficient animal strains. hNSCs survived for at least 5 months post-transplantation and differentiated into cells from all three neural cell lineages. Furthermore, hNSC transplantation facilitated cognitive recovery after TBI, even in the absence of gross histological modulation of lesion or total spared hippocampal tissue volume. Importantly, we have found an overall increase in host hippocampal neuron survival in hNSC transplanted animals and demonstrate that a correlation exists between hippocampal neuron survival and cognitive performance. Together, these findings support the use of immunodeficient rodents in models of TBI that involve the transplantation of human cells, and suggest that hNSC transplantation may be a viable, long-term therapy to restore cognition after TBI.

CHAPTER 1

Introduction

Traumatic Brain Injury: Definitions and Clinical Relevance

Broadly defined, traumatic brain injuries (TBIs) encompass any injury resulting in altered brain function or brain pathology as the result of an external force. Under this definition, altered brain function includes any period of any one of the following: a decrease in or loss of consciousness; pre- or post-event memory loss (amnesia); neurological deficits such as confusion, slowed thinking or aphasia; or vision or sensory changes, loss of balance, muscle weakness or paralysis (Management of Concussion, 2009; Menon et al., 2010), and neuroimaging techniques can be used to initially help diagnose TBI as well as for chronic monitoring of pathology progression (Lee and Newberg, 2005). An individual need not lose consciousness to sustain a brain injury. A mild TBI, or concussion, can still damage the brain at the cellular level and persons experiencing a minor concussion that does not result in hospitalization can have persistent symptoms of headaches, anxiety and/or fatigue coupled with cognitive deficits in memory, concentration and/ or attention; this constellation of symptoms is referred to as postconcussion syndrome (Hall et al., 2005b). Not all individuals who sustain trauma to the head will experience a TBI, but anyone with a history of head trauma resulting in altered brain function can be said to have sustained a TBI (Management of Concussion, 2009).

TBI is often referred to as a 'silent epidemic'; a status borne out by the fact that it ranks as the leading cause of mortality and disability in the young population worldwide (Langlois et al., 2005). Every 18.5 seconds, someone in the USA suffers a TBI (Scudellari,

2010). TBI affects upwards of 3.5 million people per year, with estimates of the associated costs of TBI to be in the hundreds of billions of dollars annually (Coronado et al., 2012). This incidence is higher than that of Alzheimer's disease, Parkinson's disease and multiple sclerosis combined (Scudellari, 2010). Critically, these figures likely underestimate the incidence and costs of TBI due to underreporting in sports- and military-related injuries, and persons who never seek healthcare (Roozenbeek et al., 2013). For example, a survey conducted in 2007 reported that 42% of respondents (584 out of 1381) who experienced some form of head trauma did not seek healthcare (Setnik and Bazarian, 2007), which suggests the actual number of people affected by TBI may be double current estimates. Furthermore, TBI-related injuries are considered to be the signature injury of military personnel who served in either the Iraq and/or Afghanistan wars, and it is estimated that nearly 60% of all casualties among soldiers admitted to Walter Reed National Military Medical Center suffer from TBI (CCCRP, 2013).

Additionally, approximately 1.1–1.4 million civilians per year were treated in US hospital emergency rooms for TBI, resulting in an average of 52,000 deaths per year (Coronado et al., 2011). For comparison, the CDC estimated the number of deaths due to motor vehicle accidents in 2011 to be 33,804. Mortality in severe TBI cases remains approximately 30% (Narayan et al., 2002), and greater than 40% of US citizens with TBI have residual disability 1-year postinjury (Corrigan et al., 2010). Prevalence estimates for long-term TBI-related disability in the US range from 3.2 million (Corrigan et al., 2010) to 5.3 million (Thurman et al., 1999) individuals; thus, nearly one out of every 50–75 American civilians are currently living with disabilities from TBI.

Underlying Pathophysiology of TBI

Primary TBI reflects the early damage that is the direct result of the physical displacement of brain structures, including contusion, vascular injury and axon shearing. Secondary TBI reflects damage that is an indirect result of the initial trauma; secondary damage therefore encompasses a variety of cellular processes that contribute to the progressive loss of cells and damage to underlying pathways over hours, days and weeks (McIntosh et al., 1996). TBI can also be classified as focal or diffuse in type, with focal TBI resulting from a direct impact to the skull (e.g., following a fall that causes compression of the brain region underlying the impact [coup] and rebound impact to the brain region directly opposite [contrecoup]) (Andriessen et al., 2010; Morganti-Kossmann et al., 2010). As a result, the location of the impact is directly related to the neuroanatomical locations of damage and resulting neurological deficits.

By contrast, diffuse TBI results from rapid acceleration–deceleration of the head, for example following a high-speed motor vehicle accident, which causes widely distributed white matter, vascular and hypoxic–ischemic damage (Andriessen et al., 2010; Morganti-Kossmann et al., 2010). Clinical studies suggest that diffuse TBI results in progressive, chronic atrophy of white matter that may continue for years postinjury (Sidaros et al., 2009). Critically, however, MRI studies suggest that 50% or more of patients with moderate-to-severe TBI exhibit a mixed etiology, in which both focal lesions and diffuse axonal injury are observed (Skandsen et al., 2010). These complex and mixed clinical observations suggest that, despite the contribution of distinct pathophysiological mechanisms to focal neuronal loss versus diffuse axonal pathology, the distinction between focal and diffuse TBI is an artificial one (Andriessen et al., 2010). This blurred distinction

highlights the importance of animal model selection, the need to replicate positive findings in more than one model, the potential need for combinatorial therapeutic approaches, and the complexities we face trying to translate more focal vs diffuse animal models to the human condition.

Modeling TBI in Rodents

Injury Models

There are a wide range of injury paradigms used to model TBI in rodents. Broadly, these are typically divided into diffuse (fluid percussion injury, weight-drop method) and focal (controlled cortical impact) injury models, although considerable pathological and functional overlap between the focal and diffuse models exists. For the purposes of this dissertation, we will focus on focal injuries induced via controlled cortical impact (CCI). For a more detailed discussion on both fluid percussion injury and the weight-drop method, see (Gold et al., 2013; Xiong et al., 2013). Additionally, more recent models of TBI exist that are increasing in popularity due to their ability to mimic war-like injuries associated with explosions and firearm-related injuries (Saljo et al., 2000; Cernak et al., 2001; Williams et al., 2005; Williams et al., 2006). However, these more diffuse injury types and methods to generate war-like injuries (blast injury model, penetrating ballistic-like brain injury) are beyond the scope of this dissertation and will not be discussed in further detail.

The studies highlighted in this dissertation (Chapters 3 and 4) both make use of the CCI model of TBI, which employs a pneumatic impact device to drive a rigid impactor to deliver mechanical energy onto the exposed and intact dura mater. This mechanical energy causes a deformation of the brain. The advantage of this model is the reproducibility and

precise control of the mechanical parameters: dwell time, velocity and depth of impact (Morales et al., 2005; Xiong et al., 2013). The first CCI was described in ferrets (Lighthall, 1988) and was later adapted for use in rats (Dixon et al., 1991) and mice (Smith et al., 1995; Hannay et al., 1999). CCI has also been described in swine and monkeys (Xiong et al., 2013). The neuropathology induced by CCI includes contusion, subdural hematoma, subarachnoid hemorrhage, edema, hypoperfusion, neurodegeneration and cavitation (Dixon et al., 1991; Kochanek et al., 1995; Hall et al., 2005a). This kind of damage occurs proximal to the mechanical impact and induces brain injury of both cortical and subcortical structures (including the hippocampus) as well as ventricular enlargement (Morales et al., 2005). The main disadvantage of the CCI model is that while some diffuse damage occurs, CCI is considered to induce focal brain injury. To more reliably model diffuse brain injury, Lateral Fluid Percussion Injury (LFPI) is typically utilized (Xiong et al., 2013).

Hippocampal neuronal loss is a common pathological feature of human TBI (Kotapka et al., 1992; Tate and Bigler, 2000; Maxwell et al., 2003; Ariza et al., 2006), and there is longstanding evidence for cognitive deficits in rodents with hippocampal neuronal cell loss (Volpe et al., 1992; Conrad and Roy, 1993). In these studies, lesions to specific hippocampal subregions or surgical procedures that result in region specific hippocampal neuron loss, including neuronal loss in the DG and CA1 regions, caused subsequent memory impairment. Additionally, similar findings have been demonstrated in genetic models of hippocampal neuronal loss (Yamasaki et al., 2007; Myczek et al., 2014) and TBI (Hicks et al., 1993). Considering then that TBI often leads to hippocampal neuron loss, which in turn has the potential to cause cognitive impairment, careful consideration must be given to the methods for detecting behavioral impairment as a result of TBI as well as for

potential improvement as a result of therapeutic treatment. Of course, one must also consider that TBI pathology (in both rodent models and also human patients) is not restricted to hippocampal neuron loss. However, the studies in this dissertation will largely be devoted to analyzing histological changes that result from a hippocampal-directed TBI impact (such as hippocampal neuron loss).

Assessments of Functional Outcome in Rodents After TBI

One of the most common and debilitating features of TBI is alterations in cognition, including confusion, memory impairments and deficits in executive function. There are a variety of tests in rodents that assess cognitive function, and these tasks will be described below. Deficits using these tasks could suggest that there is damage in the hippocampus and other brain structures involved in learning and memory. Additional behavioral assessments for motor and sensory dysfunction post-TBI can also be assessed, however for the purposes of this dissertation, we will focus the discussion on a number of cognitive (MWM, NOR, NPR) and emotional (EPM, CTA) tasks. For a more thorough review of all functional outcome measures in models of TBI, see our review (Gold et al., 2013).

In 1984, Morris developed the Morris Water Maze (MWM) task (Morris, 1984). The MWM is primarily a cognitive assessment of spatial learning and memory. Rodents are placed into a large tank filled with water and must find a platform hidden below the surface of the water. They use distal spatial cues in the testing room to find the platform (Morris, 1984; Vorhees and Williams, 2006), and spatial learning is evaluated with repeated trials to locate the hidden platform. Spatial learning refers to the process through which animals encode information about their environment to facilitate navigation through space and

recall the location of motivationally relevant stimuli. The animal must learn to use distal cues to navigate and find a hidden platform (Vorhees and Williams, 2006). Reference memory is subsequently evaluated by removing the platform. The rodent's time spent searching the space where the platform used to be is used as a measure of its reference memory (Vorhees and Williams, 2006). Reference memory represents knowledge of aspects of a task that remains constant between trials. It is a long-term memory that can last for days, weeks, months and years (Nadel and Hardt, 2011). This memory is evaluated at the end of learning, commonly by use of a probe trial 24 hours after the last acquisition day. Reversal learning of the MWM is a modification of the original testing protocol in which the platform is relocated to another quadrant (commonly in the opposite quadrant) and another four testing trials are administered for an additional 5 days. The reversal phase of MWM is considered to be more challenging than the initial spatial learning acquisition phase as it requires animals to both extinguish a previously learned location and then learn a new one (Vorhees and Williams, 2006). Multiple examples exist where experimental animals show little to no differences during spatial learning acquisition but exhibit strong deficits during reversal (Vorhees and Williams, 2014), particularly in the case of hippocampal damage. Specific deficits in MWM testing have been found in animals with damage in hippocampus, striatum, basal forebrain, cerebellum and neocortex (prefrontal cortex, insular cortex, entorhinal and perirhinal cortex) (D'Hooge and De Deyn, 2001).

Novel object recognition (NOR) evaluates nonspatial hippocampal-mediated memory. Animals are allowed to explore two identical objects in a chamber for a predetermined amount of time and then, after an intertrial interval, are placed back into the chamber with one familiar object and one novel object. The times spent with the novel

and familiar objects are recorded. Control animals will spend more time exploring the novel object. Brain structures implicated in this task are the dentate gyrus, the CA1 and CA3 regions of the hippocampus (Zhao et al., 2012), and the perirhinal cortex (Ennaceur et al., 1996).

Novel place recognition (NPR) evaluates spatial hippocampal-mediated memory. Animals are allowed to explore two identical objects in a chamber for a predetermined amount of time and then, after an inter-trial interval (typically lasting 24 hours for tests of long-term memory), are placed back into the chamber with two identical objects, but one has been moved to a new location. Control animals will spend more time exploring the object in the new location. Performance on this task is heavily dependent on hippocampal integrity, as bilateral lesions encompassing as little as 30-50% of dorsal hippocampal volume were sufficient to induce spatial memory impairment (in contrast, lesions encompassing 75-100% of dorsal hippocampus were required for object recognition deficits to become apparent) (Broadbent et al., 2004).

The elevated plus maze (EPM) is a task that assesses anxiety (Pellow et al., 1985; Walf and Frye, 2007). The maze itself is cross-shaped, with two open arms and two closed arms. Rodents have a preference for dark places; therefore, behavior differences are assessed by the time spent in the closed arms of the maze relative to the open arms. Animals are placed in the center of the maze facing an open arm and tracked using motion detection software. Animals displaying heightened levels of anxiety spend more time in the closed arms. The number of entries, time spent in each arm (open vs closed) and distance traveled are typically measured.

Finally, conditioned taste aversion is used as a measure of classical conditioning to determine if experimental animals exhibit impairment in associating a noxious event (LiCl-induced gastric malaise associated with saccharin consumption) with an everyday activity (water/saccharin consumption) (Garcia et al., 1955; Welzl et al., 2001). Several brain regions have been implicated to be involved with taste aversion-related behavior, including the amygdala and insular cortex.

Stem Cell Therapy as a Possible Treatment Strategy

It is clear that TBI in humans causes a wide range of damage and deficits, and that TBI is very heterogeneous. Basic and preclinical animal research attempts to model aspects of TBI pathology using different injury paradigms and measuring different types of motor and cognitive outcomes. Common to most injury paradigms are two of the main pathological hallmarks of TBI: neuronal cell death and white matter damage. Basic and preclinical TBI research has focused heavily on strategies to attenuate the early expansion of damage. However, although even a modest improvement in outcome for patients would have significant quality of life benefits and financial benefits, no therapy has yet to be found to improve outcomes in TBI patients. To date, there have been 131 Phase II or III clinical trials completed or ongoing in the USA for a drug or procedure (e.g., hypothermia or hyperbaric oxygen) to treat TBI; yet, there are still no US FDA-approved therapies for TBI (ClinicalTrials.gov). This failure rate has highlighted the necessity of re-evaluating both clinical translation strategies and preclinical models.

The rapid progression of stem cell research in the last 15 years has opened a new aspect of regenerative medicine research and clinical translation for neurological disease

and injury. Stem cell or other cellular therapy trials have either been completed or are underway for the treatment of patients with Batten disease, spinal cord injury, Pelizaeus–Merzbacher disease (a fatal neurodegenerative disorder of myelin), amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, ischemic stroke, and TBI (De Feo et al., 2012). In the context of TBI, the transplantation of bone-marrow derived mesenchymal/ stromal cells has been extensively studied (Li and Chopp, 2009; Aertker et al., 2015) and several clinical trials utilizing human mesenchymal stem cells have been approved and are upcoming (NCT02525432, NCT02416492), ongoing (NCT02028104, NCT01851083) or have been completed (NCT00254722, NCT01575470). Stem cell approaches to regenerative medicine for CNS disorders are thought to proffer several broad mechanisms of action: immune-modulation of the host environment; trophic factor secretion in the local microenvironment; and/or underlying functional integration with the injured host following terminal differentiation of neural stem cells (NSCs) into neurons, astrocytes and/ or oligodendrocytes. Critically, these mechanisms need not be mutually exclusive. In our experience, human NSCs (hNSCs) have the potential to simultaneously target neuronal replacement and white matter repair, to replenish lost cells following injury and/or to restore myelination of demyelinated axons (Cummings et al., 2005; Hooshmand et al., 2009). However, the usefulness of stem cell therapy for treating injury and disease is dependent upon both the reproducibility of cell behavior across multiple *in vitro* and *in vivo* studies as well as the clinical relevance of the cell population – that is, will the cells be safe to use in potential clinical applications?

Methods to Improve the Reproducibility and Clinical Relevance of Human Stem Cells

Current methodologies for the culture of embryonic and neural stem cells, regardless of their origin (mouse or human), typically rely on undefined media containing bovine serum and substrates derived from mouse sarcoma cells (Matrigel) or porcine gelatin. Various trophic factors are produced from these undefined reagents that support cell viability, but there is often lot-to-lot variance that could lead to variable results. Additionally, research has shown that human cells cultured in non-human conditions incorporate non-human proteins (for example, the non-human sialic acid Neu5Gc) that have the ability to elicit an immune response when exposed to human serum (Martin et al., 2005). This data, combined with the increased risk of contamination of clinical cells lines with unknown animal bacteria, viruses, or prion proteins, has led to increased efforts to precisely define and "qualify" all of the components in commercially available cell culture media and substrates, as well as to begin creating media and substrate formulations that contain only human or recombinant proteins ("Xeno-Free" or XF cell culture). Using defined, XF culture conditions has the potential to reduce experimental variability and will push the stem cell field towards a common cell culture baseline(s). However, due to the novelty of these new cell culture reagents, only a few studies to date have assessed the feasibility of culturing human embryonic stem cells (hESCs) in XF conditions long-term without the occurrence of any genomic or karyotypical abnormalities (Ellerstrom et al., 2006; Rajala et al., 2007; Bergstrom et al., 2011; Tannenbaum et al., 2012), and even fewer studies have been performed culturing human neural stem cells (hNSCs) in such conditions (Swistowski et al., 2009).

Additionally, sufficient expansion of resulting hNSCs to high numbers in a clinically relevant time frame has proven to be difficult, and transplantation of pluripotent or non-

fully neuralized cells has the potential to result in tumor or teratoma formation. To address these potential complications, we have hypothesized that by using a combination of a neuralization protocol that generates early stage hNSCs (Ebert et al., 2013), cell sorting to enrich for cells expressing CD133 (a cell surface epitope known to be expressed on multiple stem cell lineages) and remove cells expressing CD34 (a surface epitope expressed on hematopoetic and endothelial cells) (Uchida et al., 2000), and media and substrate conditions to promote early neural growth and prevent differentiation, we can selectively propagate a highly proliferative, enriched population of early stage hNSCs or human neuroepithelial-like stem cells in culture. Additionally, by using cell sorting to enrich for neural stem cells (CD133+) and remove non-neural lineage cells (CD34-), we predict that CD133+/CD34- sorted hNSCs will have a reduced risk for teratoma formation, which is a concern with any cell type derived from embryonic sources. Therefore, one of the main goals of this dissertation was to develop methods to generate hNSCs from hESCs in a manner that is both more clinically relevant (XF culture) and enables the propagation of cells with a higher proliferative capacity, and to do so without increasing the risk for induced karyotypic abnormalities *in vitro* or teratoma formation *in vivo*.

Predictive Models of TBI and Assessment of Human Stem Cell Therapies

While stem cell therapy strategies for TBI may offer the possibility of a new and mechanistically combinatorial approach, testing donor cell populations in animal models will add to the complexity of the criteria necessary for pre-clinical animal models with good predictive validity for clinical translation. In particular, at least two conditions would need

to be met for any relevant preclinical model to enable evaluation of the success or failure of a therapeutic stem cell approach:

First, a model in which sufficient engraftment and survival of donor human cells can be achieved to reliably test safety and efficacy across a xenotransplantation barrier will be necessary. We suggest that in a niche amenable to cell engraftment and survival, the number of animals demonstrating engraftment (that is, the presence of surviving human cells) should approach 100% of those transplanted. Furthermore, a microenvironment that enables maximal theoretical survival, as well as proliferation of transplanted stem cell populations, will be most informative, particularly for safety and tumorigenesis, but also for disease modifying activity (efficacy) and mechanism of action. At present, achieving this level of engraftment and cell survival in preclinical neurotransplantation studies will likely require immunodeficient animal models, or dramatically improved methods of achieving adequate immunosuppression (Anderson et al., 2011). It should be noted that evaluation of survival is complex, and quantification of the number of surviving cells at the end of a study is not informative about how that number came to be; the relative contributions of cell death and cell proliferation at different intervals post-transplantation will clearly both play a role in the end result. Accordingly, the initial number of transplanted cells, the number of surviving cells at an early timepoint (days) post-transplantation, and the number of surviving cells at the termination of the experiment should all be considered. In some cases it may also be appropriate to carefully consider the accuracy of the initial cell bolus delivered and the degree of early loss of transplanted cells (e.g., via assays for colocalization with apoptotic markers such as activated caspase-3).

Second, a model in which sufficient time duration is allotted from transplant to assessment to allow for the evaluation of potential functional impact is necessary, so that either improvement (efficacy) or determent (safety) can be measured. At a minimum, reproducible long-term engraftment and cell survival should be quantitatively assessed no earlier than 4 weeks post-transplantation, a time at which both the acute and delayed immunorejection phases would have been initiated. Engraftment should be reported for the entire study cohort (i.e., animals should not be excluded from analysis of these variables). For safety/tumorigenesis studies, post-transplantation analysis should be extended based on the disease indication planned for therapy; in the absence of clinical data with an identical candidate cell population, the concept of maximal theoretical engraftment should include long-term studies encompassing the lifespan of the animal model. While some immunodeficient animal models exhibit a shortened lifespan, others can be maintained to establish long-term safety data. In the case of TBI, the persistence of sustained deficits that can be reliably measured are an especially critical variable in this context. Human donor cells, in particular, may require up to several months to proliferate, migrate, differentiate and integrate *in vivo*; the time required for these processes can be anticipated to be an essential variable in determining the effect (or not) of donor cells on functional integration.

Improving the Survival of Transplanted Human Neural Stem Cells

Transplantation of stem cells into the diseased or injured CNS allows a unique replacement therapy not afforded by pharmacological agents. Stem cells can provide benefit by differentiating and integrating into the host to restore functional and behavioral deficits that result from the loss of host CNS cells (Young et al., 2000; Cummings et al., 2005;

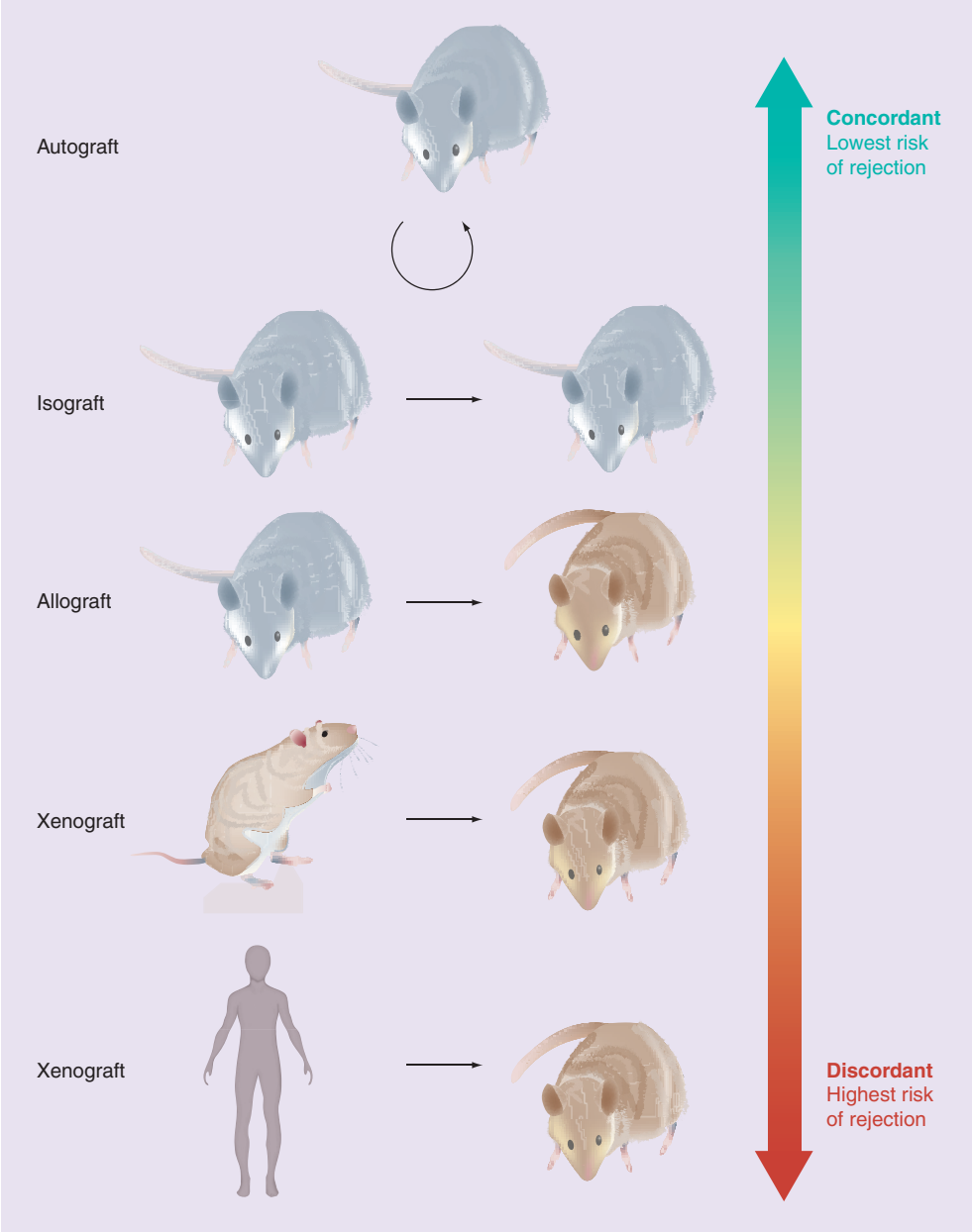

Figure 1.1. Diagram of the types of transplantation, from autografts to xenografts. As the level of donor to host increases (from concordant autograft to discordant xenograft), so does discordance and associated increased risk of immunorejection.

Yan et al., 2007). However, stem cells can also provide trophic support or deficient factors to the host tissue (Ourednik et al., 2002; Ishii et al., 2006), or modulate the immune/ inflammatory environment (Wang et al., 2014), to reduce cell loss or potentially promote host regeneration/plasticity mechanisms to restore function. In some cases, the benefits of

stem cell transplantation may derive from the short-term neurotrophic/neuroprotective effects during the acute phase postinjury/transplantation. However, the risk:benefit ratio of a cellular therapeutic that is neurotrophic/neuroprotective in nature in a human clinical population may not be as advantageous due to increased risk factors to the patient deriving from this method of delivery. These may include tumorigenesis and graft rejection. This is especially true when alternatives that offer similar mechanistic recovery are available, such as conditioned media, neutralizing antibodies, or neuroprotective pharmacological approaches. Thus, we focus here on mechanisms of action in which long-term stem cell engraftment and survival are of critical importance to the regenerative medicine field.

Transplantation Paradigm and Predictive Pre-Clinical Models

The success of laboratory and clinical transplantation is largely defined by the immunological compatibility between donor and host tissue/cells (Figure 1.1). Autografts are transplants where the donor tissue comes from the recipient. A common example of an autograft is when skin or bone is taken from one part of a patient's body to reconstruct another (Henry et al., 1961). Syngeneic transplants, or isografts, are those in which the donor and recipient are either genetically identical or sufficiently identical to allow for complete immunological compatibility. An example of a syngeneic graft is a kidney transplant between identical twins (Murray et al., 1958). Allografts are transplants where the donor and recipient are nongenetically identical members of the same species. Differences in MHC antigens, specifically HLA in humans, determine the successful integration of the graft. However, it is known from organ transplant research that MHC matches, or near matches, reduce the likelihood of graft rejection (Turner, 2004).

Regardless of the level of histocompatibility, immunosuppression is required to overcome the immune response against alloantigens. Finally, a xenograft is a transplant in which the donor and recipient are from different species. Transplantation between closely related species, such as mice and rats, is classified as a concordant xenograft. Transplantation between distantly related species, such as humans and rodents, is classified as a discordant xenograft. The level of immune response, and hence the risk of an immunorejection response, increases in magnitude when moving from an autologous transplant, to a syngeneic transplant, to a matched allograft, to a near-matched allograft, to an unmatched allograft, to a concordant xenograft and finally to a discordant xenograft (Figure 1.1). Therefore, the type of transplantation paradigm is a crucial factor in establishing a clinically relevant model system for stem cell therapy.

Informative model systems that have good predictive value of the human clinical transplantation setting are necessary to increase the chances of success in translating advances in stem cell therapy of neurological diseases from bench to bedside. From a stem cell transplantation perspective, there are two major components of a model system: the host species in which the neurological disease is being studied and the host species from which the stem cells are derived. Clearly, from both an ethical and regulatory standpoint, human subjects are not an appropriate starting point for testing stem cell therapies. However, there are a wealth of animal models that closely mimic some of the pathological and behavioral deficits associated with different neurological disorders and types of neurotrauma. While some groups have performed experiments in larger animal models such as nonhuman primates (Iwanami et al., 2005; Redmond et al., 2007; Pluchino et al., 2009), the vast majority of preclinical research using human stem cells is conducted in

rodent models due to the lower cost, a paucity of nonhuman primate models of neurological disease and injury (versus the abundance of transgenic/knockout mice), and difficulty of achieving adequate immunosuppressive regimens in nonhuman primates (Haustein et al., 2008; Cook and Tymianski, 2012; Daadi et al., 2014; Kwon et al., 2015). In fact, the latter may be such a significant barrier that, in many cases, stem cell transplantation into nonhuman primate models cannot practically be employed to inform clinical translation. Therefore, for the purposes of this dissertation, we will focus on rodent models of neurological disease and trauma.

If we accept rodent models as the basis for the study of neurological disease/trauma, what is the most appropriate stem cell source: human stem cells or animal stem cells? A large advantage to using rodent stem cells for preliminary proof-of-concept studies is that researchers can perform syngeneic or allotransplants, which can bypass many of the immunological hurdles of xenografts and more closely mimic some aspects of the clinical setting (i.e., matched human tissue grafted into a human patient). Additionally, rodent NSCs differentiate more readily and mature faster than hNSCs, thus reducing the time needed to obtain results (Wachs et al., 2003). However, while nonhuman cells can provide preliminary proof-of-concept data, the proposed population of human cells must be tested in preclinical studies for regulatory submission in optimal and appropriate animal models. Furthermore, long-term safety data must be established using the clinical grade of stem cells in the target tissue and in potentially both naive and disease/injury conditions. Consequently, there is a need in the regenerative medicine field for preclinical model systems with good predictive value; this need necessitates the use of human stem cells.

In the case of a discordant xenograft, such as human stem cells into a mouse or rat host, the main hurdle is avoiding or sufficiently minimizing the rejection response by the host immune system in order to achieve successful engraftment and survival of the transplanted human stem cells. Moreover, the presence of an active immunorejection response itself may significantly alter the efficacy, and critically, the safety profile of the cell therapy candidate. In this regard, a valid assessment of the safety profile can be argued to be particularly dependent on conditions that enable or encourage maximal theoretical engraftment and cell survival. In the absence of maximal theoretical engraftment conditions, a valid analysis of the tumorigenic potential of a cell therapy candidate may not be possible. An example of this would be the failure to develop teratomas after embryonic stem cell transplantation into immunocompetent versus immunodeficient hosts (Przyborski, 2005; Dressel et al., 2008). However, in certain instances (such as in some animal models of Multiple Sclerosis or Alzheimer's disease), immune cell activity may be a critical disease component or process and therefore the use of immunodeficient animals (or high dose immunosuppression) must be carefully considered as use may further complicate study design and data interpretation. Overall, by employing animal models that recapitulate the key elements of human pathogenesis, permit sensitive evaluation of disease-modifying activity and permit, when possible, successful engraftment and long-term survival of transplanted human stem cells, researchers can improve the predictive validity of preclinical safety and efficacy studies, and the likelihood of success of translation to clinical trials.

Achieving Transplant Engraftment and Survival Across the Xenobarrier

In accordance with the immunological barrier presented by xenotransplantation, several approaches have been utilized in an attempt to achieve high levels of transplant engraftment and cell survival in the organ and bone marrow transplantation fields. First, high-dose combinatorial treatment with multiple conventional pharmacological immunosuppressant drugs. High dose and combinatorial pharmacological immunosuppression has associated toxicity concerns in human (de Mattos et al., 2000; Wu et al., 2010) and animal models (Dumont et al., 1992; Kochi et al., 2000; Yamauchi et al., 2004). A key question to be considered is whether there may be exogenous, alternative and/or unanticipated effects of conventional immunosuppressant agents on transplanted cell populations or the disease process itself. In this regard, multiple studies have also shown that immunosuppressive agents can interact with, and influence, various cell populations, altering cell proliferation, fate, migration and perhaps secretomes (Song et al., 2006; Isomoto et al., 2007; Wang et al., 2008) as well as promote histological and behavioral benefit in rodent models of TBI (Scheff and Sullivan, 1999) and Alzheimer's disease (Taglialatela et al., 2009).

Second, humanized rodent models have been developed to lower the xenotransplantation barrier. Humanized mice or mouse–human chimeras are immunodeficient animals that are reconstituted with human-derived hematopoietic cells or tissues to minimize HLA mismatches with subsequent human-derived cell populations (Shultz et al., 2007). While in many ways humanized mice may be considered the ultimate goal for regenerative medicine research, their use is significantly confounded by several major constraints, including the low efficacy of immune reconstitution, the time required

for generation of animals, specialized equipment and significant cost. Thus, humanized rodent models of cell transplantation are rarely utilized.

Third, constitutively immunodeficient animal models, in which components of adaptive and/or innate immunity are compromised or deficient, can be utilized to improve the success of xenotransplantation. Immunodeficient animals provide an environment in which the immune response is suppressed endogenously, rather than via exogenous treatment, which allows for direct hypothesis evaluation without complications that may arise from the immunosuppressive treatments necessary to support sufficient engraftment in immunocompetent animals. Historically, evidence for long-term (up to 6 months post-transplant) tolerance of cellular xenografts in immunodeficient animal models is supported by experiments demonstrating prolonged survival of human fetal tissue and blood cells (Mosier et al., 1988; Lapidot et al., 1992), and later, pig and human islet cells in constitutively T-cell deficient mice and rats (Korsgren et al., 1991; Korsgren and Jansson, 1994) suggesting that a lack of functional T cells at least partially circumvents the barriers of chronic rejection. However, the survival of at least mouse–mouse allografts of embryonic stem cells transplanted into heart (Kofidis et al., 2005) or muscle (Swijnenburg et al., 2008), and human–rat xenografts of neural stem cells transplanted into the spinal cord (Yan et al., 2007) has also been shown to be significantly greater in immunodeficient models compared with immunosuppressed models. While a wide variety of immunodeficient/ immunocompromised rodents are available for xenotransplantation studies (Shultz et al., 1995; Greiner et al., 1998; Pearson et al., 2008), not all constitutively immunodeficient animal models achieve equal levels of immunodeficiency; identification of a model that is

'sufficiently immunodeficient', meaning that it achieves 100% engraftment and long-term survival, is therefore essential.

Owing to a loss-of-function mutation in the mouse *PRKDC* gene preventing full T- and B-cell development (Blunt et al., 1995), CB-17 SCID mice, which were used in the original hematopoetic stem cell xenografts performed by Mosier *et al.* in 1988 (Mosier et al., 1988), lack functional T- and B-cells (Shultz et al., 1995). However, SCID mice retain high levels of innate (NK cell) immunity (Shultz et al., 1995), which precludes complete avoidance of immune rejection (Bosma et al., 1988). To avoid the shortcomings observed in these early SCID models, alternative immunodeficient animal strains have been generated to further improve graft survival (Bernard et al., 2008). Nonobese diabetic (NOD)-SCID mice, which in addition to the T- and B-cell deficiencies of CB-17 SCID models, also display reduced hemolytic complement levels, reduced dendritic cell function and defective macrophage function (Shultz et al., 1995), as well as reduced NK cell activity (Poulton et al., 2001), have been used extensively in a multitude of different stem cell and transplantation studies with great success (Greiner et al., 1998). Furthermore, recent development (Ito et al., 2002; Ito et al., 2008) of genetic variants with nearly complete ablation of T-, B-, and NK cell activity offer even more effective options in a xenograft transplantation setting (Pearson et al., 2008). These include NOD-SCID IL2RG, and Rag2null IL2RG mice, which include a null mutation in the gene encoding the IL-2 receptor chain (IL2R) that prevents cell surface signaling to several interleukins as well as NK cell differentiation (Cao et al., 1995). Additionally, larger rodent models lacking certain components of the immune response also exist and may be utilized in experiments where smaller laboratory mice are not an appropriate choice; the principle example is the athymic nude (ATN) rat, which lacks

a normal thymus and functionally mature T cells (Rolstad, 2001). However, caution should be exercised when considering nude rodent models, as evidence suggests normal to increased levels of NK cell activity (de Jong et al., 1980), which may be sufficient to induce graft rejection (Lin et al., 1997; Rolstad, 2001). Accordingly, selection of an immunodeficient mouse or rat model should be considered based on the known combination of deficits in the immune response and resulting engraftment characteristics.

The studies highlighted in this dissertation (Chapters 2 and 3) utilize both immunodeficient NOD-SCID mice as well as ATN rats. For studies assessing transplanted cell engraftment and teratoma formation potential, NOD-SCID mice have been used to maximize engraftment and survival potential (Anderson et al., 2011). However, for studies measuring cognitive behavior after TBI and subsequent human stem cell transplantation, ATN rats have been utilized based upon previous research demonstrating cognitive behavioral deficits after hippocampal damage in ATNs (Acharya et al., 2009) and other studies suggesting that cognitive behavioral deficits may be more reliably detected in rats versus mice (Abbott, 2004; Snyder et al., 2009). Although the studies discussed in this dissertation will not directly compare transplanted cell survival between these two immunodeficient rodent models, we have previously found evidence for robust human cell engraftment and survival in ATN rats (Piltti et al., 2013a, b), confirming the feasibility of the studies planned.

Evaluation of Engraftment and Cell Survival for the Xenogeneic Transplantation of Stem Cells in Animal Models of CNS Injury and Disease

To either support or refute the hypothesis that the use of immunodeficient animal models will result in greater levels of transplanted human stem cell survival, we conducted a comprehensive review of the literature regarding engraftment and cell survival with xenografts in the CNS. We focused our literature review on studies xenotransplanting human cells into rodent models of neurological diseases/trauma or into the normal brain. For purposes of this review, we define 'engraftment' as the percentage of animals that demonstrate surviving cells (total number of animals at sacrifice with human cells still present divided by the total number of initially transplanted animals multiplied by 100). In parallel, we define 'cell survival' as the total number of cells present at sacrifice. The total number of cells must have been assessed either via unbiased stereology (typically via optical fractionator) or bioluminescence for the percentage cell survival report to be included in our analysis. Unbiased stereological techniques permit rigorous quantitative analysis of tissue, including accurate volume-corrected estimates of cell number; because changes in tissue and structure volume due to disease/injury pathogenesis can be a significant experimental confound, stereological analysis is the gold standard for determining cell number, lesion volume and other variables vulnerable to these artifacts (see (Coggeshall and Lekan, 1996; Schmitz and Hof, 2005)) for a review of the use of stereology in neuroscience). However, the accuracy of stereological cell quantification is only as good as the methods used for cell detection (for example, detection of human cells using antibodies specific for human cellular epitopes). In our experience, we have encountered variability in the fidelity of human specific antibodies when analyzing *in vivo* cell engraftment of transplanted non-neural lineage differentiated human cells after spinal cord injury (Hal Nguyen, unpublished findings). For this reason, human cell engraftment

may need to be verified using multiple "human specific" markers. Bioluminescence also permits quantitative analysis of cell survival within tissue, however, there are two critical limits for cell detection. First, sensitivity; while stereology permits an estimate of total cell number based on the detection of every cell visualized by immunoperoxidate, immunofluorescence, or promotor driven fluorescence in a transplanted tissue, bioluminescence has a clear threshold for detection that is affected by many factors, including transplantation site/depth. In the neural stem cell transplantation field, at least one study has demonstrated that the number of luciferase-expressing cells necessary to generate a detectable bioluminescence signal is in the order of 1000 (Takahashi et al., 2011). Coupled with this detection threshold limit, the propensity of transplanted cell populations for migration will significantly affect the accuracy of total cell survival quantification by bioluminescence. Finally, the long-term stability of luciferase expression has not been established, and decrements in signal may, in some cases, result from promoter downregulation (Takahashi et al., 2011). Owing to the limitations of

Table 1.1. Analysis of 132 unique papers reporting xenotransplantation of human stem cells into the CNS.

	Normal brain (n = 6 papers)			Acute/traumatic (n = 94 papers[‡])			Chronic/atraumatic (n = 32 papers)		
	IC with IS	IC with no IS	ID model	IC with IS	IC with no IS	ID model	IC with IS	IC with no IS	ID model
Total number of papers	3	2	1	45	37	14	21	7	4
Number reporting animals engrafted (%)	3	0	1	12	7	9	6	4	1
Number reporting cell survival (%)	2	1	1[†]	9	1	4	6	2	1
Number reporting engraftment and survival	2	0	1	1	0	4	1	1	1
Number reporting neither	0	1	0	28	30	5	9	2	3
Average number of animals engrafted (%)	78 (n = 3)	N.R.	100 (n = 1)	85 (n = 12)	71 (n = 7)	95 (n = 9)	81 (n = 6)	89 (n = 4)	87 (n = 1)
Average cell survival (%)	53 (n = 2)	8 (n = 1)	N.A.[†]	17 (n = 9)	35 (n = 1)	263 (n = 4)	83 (n = 6)	34 (n = 2)	N.A.[§]

[†]One immunodeficient paper reported a 10% cell survival via bioluminescence, but detection sensitivity is not comparable to histology.
[‡]94 unique papers, one of which includes IC animals with and without IS, and one includes IC animals in one group and ID animals in another group. These duplications are subtracted from the total. Hence, 45 + 37 + 14 = 96 – 2 = 94.
[§]One paper quantified survival stereologically within a restricted region while cells had migrated beyond that region, hence total cell survival is unknown.
IC: Immunocompetent; ID: Immunodeficient; IS: Immunosuppression; N.A.: Not applicable; N.R.: Not reported.

bioluminescence in providing accurate total cell survival quantification, papers using this method of quantification have been included in the overall table and in the calculation of percentage engraftment, but have not been included in the calculation of percentage cell survival.

We have previously conducted and published an extensive literature review of the CNS xenotransplantation literature to determine the extent to which transplanted human cells engrafted and survived in immunocompetent or immunodeficient rodent models of neurological diseases/trauma or into the normal brain (Anderson et al., 2011). Literature searches for this analysis of xenotransplantation in the CNS were performed in January 2011 with the keywords 'human stem cell' in combination with other keywords in series: 'transplantation', 'brain', 'CNS', 'spinal cord', 'spinal cord injury', 'stroke', 'middle cerebral artery occlusion', 'ischemia', 'brain injury', 'brain trauma', 'multiple sclerosis', 'Parkinson disease' and 'Huntington disease'. Additional references were added when found cited in the initial round of papers retrieved via MedLine. No papers were excluded from our analysis, *a priori*. Using these criteria, we found 133 unique, relevant papers. It should be noted that the primary focus of any given paper need not have been the key variables discussed in this article (i.e., engraftment and cell survival). Rather, many papers compared a cell line with and without an experimental treatment or other component in an injured environment, or the effects of a cell line on functional outcome, and did claim to assess either engraftment or quantify total surviving cells. We grouped these papers into three primary categories based on common features of the model: models of normal neonatal or adult brain; models of acute/traumatic injury (SCI, traumatic brain injury or stroke); and models of chronic/atraumatic injury (Parkinson's disease, Alzheimer's disease,

Huntington's disease, amyotrophic lateral sclerosis, allodynia or demyelination). Within each primary category, references were subdivided into those using immunocompetent animals with immunosuppression, those using immunocompetent animals without immunosuppression, and those using immunodeficient animals. A summary of the primary categories and the number of papers using immunocompetent versus immunodeficient animals is shown in Table 1.1 For a more complete review, including detailed descriptions of characteristics of individual papers, see our review (Anderson et al., 2011).

Table 1.1 shows that of the 133 unique papers, six papers examined xenotransplantation in normal brain (5%), 95 papers examined xenotransplantation in acute/traumatic models (71%), and 32 papers examined xenotransplantation in chronic/ atraumatic models (24%). A total of 79 papers (59%) reported neither engraftment nor survival, while only 11 papers (8%) reported both engraftment and survival. For example, in the primary category 'acute/traumatic', 37 out of 95 papers (40%) used no immunosuppression in immunocompetent animals. Seven of these 37 papers reported the percentage of animals engrafted, only one reported the percentage of cell survival based on stereology, no papers reported both engraftment and percentage of cell survival, and 30 reported neither variable. In this category (acute/traumatic), the one paper to report cell survival (based on stereology) in a stroke model without the use of immunosuppression reported that only 35% of the initial cell dose survived 8 weeks post-transplant across four treatment groups (Lee et al., 2009).

Several key conclusions can be drawn from our review of the literature in 2011. First, the majority of CNS xenografts have used immunocompetent animals coupled with immunosuppression (n = 69 or 52%). The next most common paradigm is to use

immunocompetent animals and no immunosuppression (n = 47 or 35%). Only 19 papers (or 14%) used immunodeficient animals in CNS xenograft studies using human cells. Second, the percentage of animals engrafted (when reported) is usually highest in immunodeficient animals. Third, the paucity of studies in the normal CNS and chronic/ atraumatic cohorts that have employed immunodeficient models and quantitatively assessed engraftment/cell survival makes acute/ traumatic models the only category in which these variables can be compared between immunocompetent and immunodeficient animals, and makes this the most robust category from which to draw relative conclusions for xenotransplantation success. In the acute/traumatic/cohort, the highest percentage of cell survival is in immunodeficient animal models (263%, n = 4). Fourth, although the studies available for comparison are limited, the data suggest that an uninjured niche (normal brain) may be no better than an injured niche in terms of engraftment or cell survival. Finally, caution should be exercised in interpreting papers that report surviving cell numbers. Many papers extrapolate total cell number from a limited number of histological sections. Moreover, even when systematic random sampling and stereological assessment of total cell number is performed, the number of animals assessed can be insufficient to yield interpretable numbers. For example, Suzuki *et al.* reported the stereological assessment of total cell number in an amyotrophic lateral sclerosis model where 114% of the initial dose of cells was detected 6 weeks post-transplant; but quantification was performed in only one animal (Suzuki et al., 2007).

Overall, this review of 133 xenotransplantation papers shows that the field of regenerative medicine has focused heavily on the administration of immunosuppressive drugs such as cyclosporin A alone in immunocompetent animals as a strategy for proof-of-

concept experiments, resulting in both poor engraftment and low to very low cell survival (when reported). Furthermore, this summary shows that xenografted stem cells retain proliferative capacity in immunodeficient versus immunosuppressed models within the acute/traumatic category. Combined with our historical survey of the broader xenotransplantation field, this analysis clearly suggests that it will be necessary to administer a multi-modal course of pharmacological immunosuppression to achieve meaningful engraftment of a transplanted human cell population when using immunocompetent animals. Alternatively, immunodeficient animals yield much higher engraftment and cell survival numbers than using immunocompetent animals (with or without immunosuppression).

Future Perspective in Achieving Improved Transplanted Human Stem Cell Survival in Animal Models

It seems evident that, ideally, preclinical testing of safety and efficacy should have the goal of achieving a human–rodent xenograft that is as comparable as possible to the human clinical setting (i.e., a human–human allograft). We suggest that preclinical animal models in which the number of surviving cells is a fraction of the number contained in the initial transplant cannot provide informative data regarding proliferation potential, and therefore, cannot provide adequate data for predictive validity in the human clinical setting. The limits of such data are particularly relevant for establishing a risk versus safety profile for a candidate clinical cell therapeutic, as tumorigenesis is known to be dependent on both dose and cell survival, and is greatly attenuated in immunosuppressed immunocompetent versus constitutively immunodeficient animal models (Przyborski, 2005; Dressel et al.,

2008). However, while it might be argued that efficacy data would only be enhanced by increased cell survival, therefore supporting the use of immunosuppressed immunocompetent animal models, we suggest that this is an assumption with little or no supporting empirical data. Cells respond to both the microenvironment into which they are transplanted, and the conditions of that microenvironment. The potential effects of a microenvironment in which there is an active immune response, or conversely, an increase in cell survival due to a lack of immune-mediated cell death, on cell fate and migration are essentially unknown, and are likely to be different for each candidate clinical cell therapeutic. Furthermore, the potential for an active immunorejection response to contribute to disease modifying activity cannot be ruled out. Owing to the known effect of dose and immune rejection on tumorigenesis, and the unknown effect of dose and immune status on fate, migration and/or disease modifying activity, simply scaling up the initial cell dose administered in an immunosuppressed immunocompetent animal model is not adequate to address either tumorigenesis or efficacy. Accordingly, preclinical studies should be designed to reduce immunorejection of transplanted cells in order to optimize cell engraftment and survival, and preclinical transplantation models should seek to achieve maximal theoretical engraftment in order to provide informative safety and efficacy information. We therefore suggest that immunodeficient animal models should be the model of choice for preclinical testing of safety and efficacy for candidate clinical cell therapeutics.

There are two principal issues in considering this approach. One key question is whether there are exogenous, alternative and/or unanticipated effects of conventional immunosuppressant agents on the transplanted cell population. Many investigators have

used this rationale to continue with experiments in xenograft models using pharmacological immunosuppressive therapy in immunocompetent animals. However, we suggest that it is time for the field of regenerative medicine to re-evaluate this concept. We suggest that a more informative approach would be to establish data from two models. First, investigation of engraftment, tumorigenesis, fate and migration in immunodeficient animal models treated with the planned clinical immunosuppressive therapy. In this paradigm, any potential direct effects of immunosuppressant treatment on the candidate clinical cell population would be more likely to be revealed, given the increased overall cell survival, and reduced likelihood of the inadvertent selection against either the primary stem cell population, or precursor/progenitor populations, providing an improved assessment of tumorigenesis and safety. Recently, the effects of immunosuppressive drugs (cyclosporine A, tacrolimus, and rapamycin) on fetal hNSC survival, proliferation, and fate were assessed both *in vitro* and *in vivo* after spinal cord injury (Sontag et al., 2013). In this study, immunosuppressive drugs altered *in vitro* proliferation and differentiation but no differences were seen *in vivo*, further supporting the validity of immunodeficient animal use. Second, the effect of immunosuppressant withdrawal on target organ integrity, engraftment, tumorigenesis, fate and migration in an immunocompetent animal model treated with the planned clinical immunosuppression therapy for a transient period of time (similar to what would be prescribed in the clinical trial protocol) following cell transplantation.

A second key question is whether predictive models of neurological disease/injury for testing cellular therapeutics can be established in immunodeficient versus immunosuppressed models. It is increasingly clear that there is a role for both the innate

and adaptive immune responses in many types of neurological disease and injury. In the case of autoimmune diseases of the CNS, this role can be pivotal to the development of pathology (e.g., the generation of autoreactive T cells to myelin epitopes in multiple sclerosis); while the specific role of adaptive immune responses and T-cell activation are less clear, it appears certain that Alzheimer's disease, Parkinson's disease, stroke, traumatic brain injury and SCI all include an inflammatory component in their pathogenesis (Alexander and Popovich, 2009; Rivest, 2009; Becker, 2010; Perry et al., 2010; Qian et al., 2010; Carty and Bowie, 2011). Furthermore, in the case of traumatic injury models such as SCI, access of the immune system to the CNS is greatly enhanced by break-down in the blood–brain barrier, and there may be profound, prolonged and diverse effects of immune activation on pathogenesis and functional outcome (Beck et al., 2010). Understandably, the generation of transgenic neurological disease models backcrossed onto constitutively immunodeficient mouse strains is a difficult process. Furthermore, the effect of constitutive ablation or attenuation of T-cell and NK cell responses associated with immunodeficient models, such as the NOD-SCID mouse, on innate immune responses, inflammation and the essential characteristics of lesion pathogenesis would have to be tested as a part of validating a predictive animal model (Luchetti et al., 2010). At least in the case of traumatic SCI, the host macrophage/microglia response, neutrophil response and evolution of the central lesion are overtly unaltered in NOD-SCID mice in comparison with other mouse strains (Luchetti et al., 2010). As noted earlier, however, the immune and inflammatory microenvironment, composed of a host of complement proteins, cytokines and chemokines, may affect transplanted cell populations in ways that have yet to be recognized, and the potential for an immunorejection response to influence stem cell populations is equally as

great as the potential for alteration of the immune microenvironment presented by the disease state to do so. Critically, however, in order to achieve engraftment of a candidate therapeutic human cell population at any level for the purpose of assessment of safety/ efficacy in animal models, it is necessary to impair the functional immune status of the host, particularly with regard to activation of T-cell- and NK cell-mediated immune responses; the difference between immunosuppressed and immunodeficient lies then in the fact that, at least under some conditions, treatment with

immunosuppressants is not sufficient to achieve levels of xenoengraftment that may be comparable to human–human allotransplantation.

In summary, we suggest the following criteria for preclinical safety and efficacy studies for the application of human stem cell populations in a clinical setting:

1) At a minimum, reproducible long-term engraftment and cell survival should be quantitatively assessed no earlier than 4 weeks post-transplantation, a time at which both the acute and delayed immunorejection phases would have been initiated. Engraftment should be reported for the entire study cohort (i.e., animals should not be excluded from analysis of these variables).

2) We suggest that in a niche amenable to cell engraftment and survival, the number of animals demonstrating engraftment should approach 100% of those transplanted. At present, at least in acute/traumatic models, this percentage of xenoengraftment can only be achieved in immunodeficient models.

3) For safety/tumorigenesis studies, post-transplantation analysis should be extended based on the disease indication planned for therapy; in the absence of clinical data with an identical candidate cell population, the concept of maximal theoretical engraftment should

include long-term studies encompassing the lifespan of the animal model. While some immunodeficient animal models exhibit a shortened lifespan, others can be maintained to establish long-term safety data.

4) While in actual human clinical trials, the maximal theoretical number of surviving transplanted cells is unknown, we suggest that a microenvironment that enables maximal theoretical survival, as well as proliferation of transplanted stem cell populations, will be most informative, particularly for safety and tumorigenesis, but also for disease modifying activity (efficacy) and mechanism of action. At present, this type of survival in preclinical neurotransplantation studies, at least in acute/ traumatic models, has only been achieved in immunodeficient models.

5) It should be noted that evaluation of survival is complex, and quantification of the number of surviving cells at the end of a study is not informative about how that number came to be; the relative contributions of cell death and cell proliferation at different intervals post-transplantation will clearly both play a role in the end result. Accordingly, the initial number of transplanted cells, the number of surviving cells at an early timepoint (days) post-transplantation, and the number of surviving cells at the termination of the experiment should all be considered. In some cases it may also be appropriate to carefully consider the accuracy of the initial cell bolus delivered and the degree of early loss of transplanted cells (e.g., via assays for colocalization with apoptotic markers such as activated caspase-3).

6) We suggest that studies testing the direct effects of planned clinical immunosuppressive therapy on candidate therapeutic stem cell populations be conducted

in an immunodeficient model, to rule out possible unexpected interactions under conditions of maximal theoretical engraftment.

7) Bystander effects resulting from immune activation/diversion via activation of immunorejection mechanisms may provide part or all of the beneficial effects yielded from a given cell transplantation strategy. Accordingly, if immunosuppressed immunocompetent models are used, we suggest that assessment of host-mediated rejection mechanisms should be considered in addition to quantification of engraftment and cell survival. However, *in vivo* assessment of immunorejection responses is complex, as multiple cell types, timing and locations (e.g., within the target tissue, adjacent lymph nodes or systemic immune effects) can all play a role, and an established protocol to assay for immunorejection in animal models has not as yet been developed.

The Need for Long-Term TBI Studies

Earlier in this chapter, we proposed two criteria necessary to fully evaluate therapeutic success of human stem cell transplantation strategies in preclinical animal studies of TBI. First and foremost, for the safety/efficacy of transplanted cells to be appropriately evaluated, survival and/or proliferation potential of transplanted cells must be maximized for the duration of the study (Anderson et al., 2011). Second, to evaluate therapeutic potential, there needs to be sufficient time for engrafted cell integration to effect functional outcome. Functional integration of transplanted stem cells as neurons has been demonstrated via electrophysiological measurement of neuronal firing between transplanted and host cells in the uninjured adult, immunodeficient mouse brain (Weick et al., 2011). Integration of hNSC or oligodendrocyte precursors via electron microscopic

evidence of remyelination of host axons by transplanted cells in spinal cord models (Cummings et al., 2005; Wang et al., 2013) have also been shown. In contrast to functional integration with the host, promotion of recovery due to neurotrophic support of spared host tissue is also a possibility. Transplanted stem cells have been shown to secrete both prosurvival and immune-modulatory factors *in vivo* (Yasuhara et al., 2006; Andres et al., 2011; Horie et al., 2011), which could lead to an increase in the amount of spared tissue or an increase in the health of the spared tissue, thus enabling greater neural network efficiency.

Depending on the type of human stem cell transplanted and the transplant location, the time frame for integration and terminal differentiation may vary, and in the case of human embryonic-derived NSC-transplanted brain studies may require up to 6 months (Nasonkin et al., 2009). Critically, based on our experience with hNSCs and spinal cord injury (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010; Piltti et al., 2013a), we would predict that sustained deficits post-TBI of at least 1 month, and more likely at least 2 months post-injury, are needed to allow for detection of cell transplant mediated functional effects if such effects are via integration and not via trophic-mediated mechanisms. Even in the case of either immune modulation or trophic factors, functional improvements at greater than 1 month post-injury would be desirable. As many TBI studies have focused on short-term outcomes, it is unclear which of the existing TBI models may meet these criteria to support safety and efficacy studies, particularly in the context of long-term deficits.

Evaluation of Functional Outcome and Duration of Study in Previous TBI Research

To better understand if there is an optimal combination of injury model and functional assessments that yields prolonged functional deficits, for example, ≥1 month post-TBI, we previously conducted and published a review of the literature from the beginning of PubMed indexing to 31 March 2013, in order to objectively evaluate the range of functional assessments that exhibit prolonged deficits (≥1 month) in various TBI models (Gold et al., 2013). The goal was to evaluate whether TBI models exist that enable detection of safety and efficacy in the context of stem cell transplantation strategies. We conducted ten PubMed searches with the following search terms: 'controlled cortical impact AND water maze', 'controlled cortical impact AND cognition', 'fluid percussion AND water maze', 'fluid percussion AND cognition', 'blast AND water maze', 'blast AND cognition', 'weight drop AND water maze', 'weight drop AND cognition', 'closed head injury AND water maze', and 'closed head injury AND cognition'. All papers matching these search terms were merged into one list.

Initially, 817 papers were found with these search terms, but results were then filtered for studies in rats or mice only (371 remained) that were not reviews (362 remained) and that included an uninjured/sham control group in comparison with an injured group in one or more functional assessments (45 more papers were excluded). This filtering indicates that approximately 12% of rodent TBI papers did not include sham versus injury comparisons and resulted in a total of 314 unique papers for review.

In total, 314 unique papers were reviewed, of which 101 (32%) conducted a functional test at, or after, 1 month post-injury. Of these 101 papers assessing function at ≥1 month post-TBI, 88 papers (87%) demonstrated deficits in one or more functional outcomes. Since we have suggested that even a 1 month assessment may not be sufficient

time to allow for integration of hNSCs with the host, we also evaluated the number of papers that assessed function at 2 , 3 , 6 and 12 months post-TBI. Combining papers using any injury model and any functional assessment demonstrating sustained deficits, we found 27 papers (9%), 17 papers (5%), eight papers (2.5%) and four papers (1%) at these respectively longer time points post-TBI exhibited deficits on one or more tasks.

Human Stem Cell Transplantation in Animal Models of TBI that Measure Behavior Long-Term

Of the 314 unique papers reviewed, 31 (10%) transplanted some form of human cell population (Table 1.2). However, despite the desirability of long-term studies, very few (16%) of the human stem cell transplantation studies in rodent TBI models reviewed here (five of 31 papers indicated in Table 1.2) exceeded 6 weeks duration post-transplant. Furthermore, the majority of human cell transplant studies into TBI models report transplanted cell survival under 10% or do not report on cell survival quantitatively at all.

Table 1.2. Human cellular therapy studies in rodent models of traumatic brain injury.

(Author, year)	Cell Type	Host Species	Final Time Point (post-transplant)	Cell Survival	Behavioral Assessment
(Al Nimer et al., 2004)	Fetal hNSC	Sprague-Dawley	6 Weeks	N.R.	N.A.
(Chen et al., 2009)	Human Amniotic Cells	Sprague-Dawley	1, 2, 3, and 4 Weeks	N.R.	N.A.
(Chen et al., 2011)	Human Amniotic Cells	Sprague-Dawley	2 Weeks	N.R.	Improved Rotarod
(Gao et al., 2006)	Fetal hNSC	Sprague-Dawley	2 Weeks	N.R.	Improved MWM
(Heile et al., 2009)	Immortalized hMSC	Sprague-Dawley	2, 7, and 14 Days	N.R.	N.A.
(Hung et al., 2010)	Immortalized hMSC	Sprague-Dawley	2, 7, and 14 Days	N.R.	N.A.
(Jiang et al., 2011)	hMSC	Wistar	6 Weeks	N.R.	Improved MWM, mNSS
(Kim et al., 2010)	hMSC	Sprague-Dawley	1, 2, 8, 15, 22, and 29 Days	N.R.	Improved Rotarod, mNSS
(Li et al., 2011a)	hMSC	Wistar	6 Weeks	N.R.	Improved MWM, mNSS
(Longhi et al., 2004)	NT2N Neurons	C57BL/6	4 Weeks	N.R.	Improved MWM
(Lu et al., 2007a)	hMSC	Wistar	5 Weeks	N.R.	Improved MWM, mNSS

Table 1.2. Human cellular therapy studies in rodent models of traumatic brain injury.

(Author, year)	Cell Type	Host Species	Final Time Point (post-transplant)	Cell Survival	Behavioral Assessment
(Lundberg et al., 2009)	hMSC	Sprague-Dawley	1 and 5 Days	N.R.	N.A.
(Mahmood et al., 2005)	hMSC	Wistar	3 Months	N.R.	Improved mNSS
(Muir et al., 1999)	hNT Neurons	Sprague-Dawley	2 Weeks	N.R.	No Differences
(Philips et al., 2001)	NT2N Neurons	Sprague-Dawley	2 and 4 Weeks	N.R.	No Differences
(Qu et al., 2009)	hMSC	C57BL/6	5 Weeks	N.R.	Improved MWM
(Qu et al., 2011)	hMSC	Wistar	2 Weeks	N.R.	Improved MWM, mNSS
(Skardelly et al., 2011)	Fetal hNPC	Sprague-Dawley	12 Weeks	N.R.	Improved Rotarod, mNSS
(Walker et al., 2010)	hMSC	Sprague-Dawley	3 Days	N.R.	N.A.
(Wang et al., 2012)	Fetal hNSC	Sprague-Dawley	4 Days	N.R.	N.A.
(Wennersten et al., 2004)	Fetal hNSC	Sprague-Dawley	2 and 6 Weeks	N.R.	N.A.
(Watson et al., 2003)	NT2N Neurons	C57BL/6	4 Weeks	N.R.	Improved MWM
(Zanier et al., 2011)	Human Umbilical Cord SC	C57BL/6	5 Weeks	N.R.	Improved MWM, mNSS, beam walk
(Zhang et al., 2005)	NT2N Neurons	Sprague-Dawley	4, 8, and 12 Weeks	N.R.	No Differences
(Lu et al., 2002)	Human Umbilical Cord SC	Wistar	4 Weeks	N.R.	Improved Rotarod, mNSS
(Poltavtseva et al., 2012)	Fetal hMSC and hNSC	Outbred Albino Rat	3 Weeks	N.R.	Improved Limb Function
(Vaysse et al., 2015)**	hNT2 Neurons	Sprague-Dawley	3 Months	N.R.	Improved Grip Strength
(Xu et al., 2015)**	hESC-OPC	Athymic Nude Rat	3 Months	N.R.	N.A.
(Hwang do et al., 2014)**	Fetal hNSC	Sprague-Dawley	14 Days	N.R.	N.A.
(Tang et al., 2013)**	hiPSC-NSC	Sprague-Dawley	30 Days	N.R.	Improved Rotarod, mNSS
(Lundberg et al., 2012)**	Fetal hNSC	Sprague-Dawley	1 Day	N.R.	N.A.
(Wennersten et al., 2006)	Fetal hNSC	Sprague-Dawley	3 or 6 Weeks, 6 Months	0.03-0.2%	N.A.
(Mahmood et al., 2003)	hMSC	Wistar	4 Weeks	0.5-0.6%	Improved Rotarod, mNSS
(Hagan et al., 2003)	Fetal hNSC	Sprague-Dawley	6 Days	0.12-0.95%	N.A.
(Hong et al., 2011)	Human Umbilical Cord SC	Sprague-Dawley	3 Weeks	6.77%	Improved MWM
(Nichols et al., 2013)	hMSC	Sprague-Dawley	12 Weeks	21%	Improved MWM (11-15dpi only)

*A total of 31 papers have transplanted human stem cells into a rodent model of traumatic brain injury. Only five of these papers reported a quantified cell survival, and none of them used immunodeficient rodent strains (though one publication since July 2013 has used an immunodeficient ATN rat, but cell survival was not quantified in this study**). Injury model, transplanted human stem cell population, length of study, quantified cell survival and functional assessments in treated compared with untreated animals is shown.*
*CCI: Controlled cortical impact; dpi: Days postinjury; FPI: Fluid percussion injury; hMSC: Human mesenchymal stem cell; hNSC: Human neural stem cell; hUCSC: Human umbilical cord stem cell; mNSS: Modified neurological severity score; MWM: Morris water maze; NA: Not assessed; PBBI: Penetrating ballistic-like brain injury; WDIA: Weight drop impact-acceleration. **Signifies a paper that was added after July 2013 (5 papers total)*

Of the 31 publications using a human stem cell population in a rodent model of TBI, 26 studied at 6 weeks or less post-transplant. Of these, nine did not perform functional assessments. Of the 17 studies that did perform some functional assessment, two reported no difference between controls and transplanted animals while 15 reported improvements on MWM (n = 10), mNSS (n = 8), rotarod (n = 4), beam walk (n = 1) and/or limb function (n = 1); (the total does not equal 15 as some groups used more than one task). Only two of the 15 with positive functional outcomes also reported on human cell survival; Mahmood *et al.* (Mahmood et al., 2003) with 0.5% surviving human mesenchymal stem cells (hMSCs) at 4 weeks post-transplant; and Hong *et al.* (Hong et al., 2011) with 6.8% surviving human umbilical cord stem cells at 3 weeks post-transplant. Accordingly, the lack of surviving human cells and the short duration of the majority of TBI experiments make interpretation of mechanism and capacity to exert sustained functional effects difficult. In particular, the short time course of survival and functional assessment suggest that while the positive effects seen could be due to immune-modulation and/or trophic mechanisms, cellular integration is unlikely as a mechanism (as discussed above).

Of the five studies that conducted evaluations for 6 weeks or greater, the results have been mixed in terms of human cell survival and/or functional recovery. Two studies reported partial data on long-term cell survival, but did not examine functional outcomes after such long-term engraftment. Wennersten *et al.* noted human cell survival at both 6

weeks and 6 months post-transplant of human fetal NSCs, but reported only 0.2% human cell survival at 6 weeks (determined by counting human nuclei in four sequential sections) in cyclosporin A-treated Sprague–Dawley rats (Wennersten et al., 2006). Quantification of human cells at 6 months was not performed. Approximately 5% of human cells colocalized with neuronal or astrocytic markers; no oligodendrocytes were detected. The authors noted that the hippocampus appeared more conducive to neuronal differentiation than the cortex. No functional tasks were evaluated at any time point in Wennersten's study (Wennersten et al., 2006). Nichols *et al.* transplanted retinoic acid-primed $CD133^{+}ABCG2^{+}CXCR4^{+}$ hMSCs into the lateral ventricle of male Sprague–Dawley rats 24 h after FPI; no immunosuppression was used (Nichols et al., 2013). The transplanted cells were reported to integrate within the host and differentiate into cells expressing both immature and mature neural lineage markers. However, the cells were prelabeled with a fluorescein dye (carboxyfluorescein succinimidyl ester) prior to transplantation and not detected with human-specific markers postmortem, making positive identification difficult and colocalization with neural lineage markers uncertain. Furthermore, the methods section of (Nichols et al., 2013) indicated that 500,000 cells were transplanted, while Figure 9A of (Nichols et al., 2013) shows 100,000 cells transplanted with approximately 21,000 surviving in TBI rats (21%). Finally, function on the MWM was only examined 11–15 days post-transplant, not long-term, where significance relative to uninjured controls was observed for 'primed' hMSCs but not unprimed hMSCs (Nichols et al., 2013).

Additionally, three studies reported on both engraftment/survival and long-term functional assessments. Zhang *et al.* transplanted human NT2N neurons into a FPI model of TBI in cyclosporin A-treated Sprague–Dawley rats and reported human cell survival at 12

weeks post-transplant and the presence of human synaptophysin-positive structures (Zhang et al., 2005). However, no human cell quantification was performed and there were no functional differences in cognitive or motor performance 12 weeks post-transplant between groups injected with either NT2Ns, human fibroblasts or vehicle. Skardelly *et al.* transplanted human fetal-derived neural progenitor cells via local or systemic injection into Sprague–Dawley rats 24 h after a severe CCI (2.5 mm depth, 4 m/s impact) (Skardelly et al., 2011). Cyclosporin A and prednisolone were used for immunosuppression. Functional motor improvements (by blinded observer) on both rotarod and mNSS were observed, but only for systemically administered cells. PKH-26 prelabeled human cells were detected 12 weeks post-transplant, but no human cell quantification was performed. Similarly, Mahmood *et al.* reported motor improvement (mNSS) at 12 weeks postadministration of human marrow stromal cells into Wistar rats (Mahmood et al., 2005). A total of 2, 4 or 8 million cells were administered systemically via tail vein 24 h post-CCI-induced TBI (2.5 mm depth, 4 m/s impact). All three doses of hMSCs resulted in significant improvements in mNSS, which was assessed blind to treatment. Nonstereological human cell quantification was performed in three sections per animal and showed that human cells survived 12 weeks post-injection; none were NeuN- or GFAP-positive.

Interestingly, since publication of the review described above in July 2013, twenty-seven additional papers were published describing the transplantation of human cell types into rodent models of TBI (Chang et al., 2015). Since July 2013, five studies have assessed transplanted human neural cell types in TBI models (Lundberg et al., 2012; Tang et al., 2013; Hwang do et al., 2014; Vaysse et al., 2015; Xu et al., 2015). However, while two of these studies reported the successful engraftment and survival of human pluripotent-

derived stem cells in rodent models of TBI, including the demonstration of human induced pluripotent stem cell (hiPSC) derived NSC engraftment and cell tracking (Tang et al., 2013) as well as the first demonstration of successful hESC-derived oligodendrocyte progenitor cell (OPC) engraftment in an ATN TBI model (Xu et al., 2015), to our knowledge, zero studies have been conducted to date assessing the effects of human embryonic stem cell-derived NSC transplantation in modulating cognitive function after TBI, which was one of the primary objectives of this dissertation.

Future Perspective in Conducting Long-Term TBI Studies

Overall, while two 12 week duration studies report functional improvements after human stem cell treatment, the absence of stereological quantification of the total number of surviving human cells or their terminal fates makes it difficult to associate functional gains with human cells when the extent of human cell survival and differentiation after transplantation are unclear. Given the time, expense and use of animals, we must be rigorous when designing human stem cell transplantation studies to enable sufficient cell survival and allow adequate time for terminal cell differentiation, and we must also be rigorous in our quantification and analysis so that the potential for misinterpretation, whether beneficial or detrimental, is minimized. Taken together, these studies highlight the potential for both long-term survival of transplanted human cell populations as well as the ability for transplanted cells to possibly integrate within the host and lead to functional improvements post-TBI. However, these studies also suggest that to improve the interpretability of future CNS injury/human cell transplantation studies, researchers must accurately report engraftment (the number of transplanted animals with any surviving

cells) and quantify cell survival (absolute number of cells per animal) so that comparisons of different treatments can be made, and use immunodeficient animal strains or combinatorial immunosuppression protocols whenever possible to maximize cell survival and ensure that safety/efficacy can be adequately evaluated.

Overall Summary

Together, this dissertation details methods to generate hNSCs in a manner that enables greater reproducibility and clinical relevance, and then evaluates the pre-clinical efficacy of these cells in restoring cognition in a rodent model of TBI. Furthermore, we recommend two principal criteria for testing the safety and efficacy of human donor cell populations in preclinical TBI models. First, a model in which sufficient engraftment and survival of donor human cells can be achieved to reliably test safety and efficacy across a xenotransplantation barrier will be necessary; this requirement will likely require immunodeficient animal models, or dramatically improved methods of achieving adequate immunosuppression. Second, a model in which the potential functional impact, either in terms of improvement (efficacy) or determent (safety) can be reliably measured for an extended period of time post-transplantation (≥2 months).

Keeping these criteria in mind, in the following chapters we describe studies undertaken to first develop and characterize both *in vitro* and *in vivo* a novel population of human embryonic stem cell-derived neural stem cells (Chapter 2) and then to furthermore evaluate the potential of this cell population to restore cognition in an immunodeficient rodent model of TBI (Chapter 3). Additionally, current progress on two different ongoing studies evaluating the survival and migration of transplanted Shef6 hNSCs will be discussed

(Chapter 4), including the quantification of transplanted hNSC survival and proliferation at early time points (2-days and 2-weeks) post-transplantation (as opposed to 20-week survival discussed in Chapter 3). Finally, a broad review of the state of hNSC transplantation in the field of CNS trauma will be discussed and recommendations for future studies will be presented (Chapter 5).

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CHAPTER 2

CD133-enriched xeno-free human embryonic-derived neural stem cells expand rapidly in culture and do not form teratomas in immunodeficient mice

Abstract

Common methods for the generation of human embryonic-derived neural stem cells (hNSCs) result in cells with potentially compromised safety profiles due to maintenance of cells in conditions containing non-human proteins (e.g. in bovine serum or on mouse fibroblast feeders). Additionally, sufficient expansion of resulting hNSCs for scaling out or up in a clinically relevant time frame has proven to be difficult. Here, we report a strategy that produces hNSCs in completely "Xeno-Free" culture conditions. Furthermore, we have enriched the hNSCs for the cell surface marker CD133 via magnetic sorting, which has led to an increase in the expansion rate and neuronal fate specification of the hNSCs *in vitro*. Critically, we have also confirmed neural lineage specificity upon sorted hNSC transplantation into the immunodeficient NOD-*scid* mouse brain. The future use or adaptation of these protocols has the potential to better facilitate the advancement of pre-clinical strategies from the bench to the bedside.

Introduction

Regenerative medicine strategies for central nervous system (CNS) injury and disease represent a major unmet clinical need. One approach will likely include the transplantation of human neural stem cells (hNSCs). Indeed, fetal- and embryonic-derived

hNSCs are currently in phase I clinical trials for multiple neurological disorders, including spinal cord injury (Cummings et al., 2005; Salazar et al., 2010), Pelizaeus Merzbacher disease (Uchida et al., 2012), and dry age-related macular degeneration (Schwartz et al., 2012). However, despite the promise afforded by these trials, obstacles (including a complicated FDA approval process for cell lines, difficulties expanding cell lines sufficiently for human transplantation, and tumorigenicity concerns (Germain et al., 2012) resulting from residual, non-differentiated pluripotent cells) still remain.

Future cell-based strategies using new cell lines will benefit from the use of protocols designed to produce readily expandable cell lines with robust safety profiles during the initial pre-clinical phases of research that address FDA concerns for clinical compliance. Here, we report feasible methodologies to generate highly expandable multipotent hNSCs from human embryonic stem cells (hESCs) under completely Xeno-Free (XF) and feeder-free culture conditions. Additionally, we have magnetically sorted the XF hNSCs to further enrich for a highly proliferative neural stem population (CD133+) and reduce the potential for non-neural tumor formation (Tamaki et al., 2002). Together, XF cell culture methods and population enrichment via cell sorting may offer a streamlined approach to generate more readily approvable, expandable, and potentially safer cell populations for CNS transplantation.

Materials and Methods

Human Embryonic and Neural Stem Cell Culture and Differentiation

Culture of hESC lines Shef3, Shef4, and Shef6 (University of Sheffield, UK) was established at UC Irvine in accordance with all appropriate hSCRO and IBC protocols on

mitotically-inactivated mouse embryonic fibroblasts (MEFs, EMD Millipore) and in defined media consisting of KO DMEM/F12, 20% KO Serum Replacement (KO SR), 0.1mM NEAA, 2mM GlutaMAX, 0.1mM ß-Mercaptoethanol, and 20ng/mL bFGF (All from Life Technologies). To transition cells to Xeno-Free (XF) culture conditions, all non-human animal-based components (MEFs, KOSR) were removed and replaced with human-based or recombinant alternatives including CELLstart CTS, KO SR Xeno-Free CTS, and KO SR GF Cocktail CTS (All from Life Technologies). XF hESC culture media consisted of KO DMEM/ F12, 15% KO SR Xeno-Free CTS, 2mM GlutaMAX, 1X KO SR GF Cocktail CTS, 0.1mM ß-Mercaptoethanol, and 20ng/mL bFGF. Cells were manually split every 4-7 days upon reaching ~90% confluence.

For neuralization, an adapted version of a previously published "EZ-sphere" based neuralization protocol (Ebert et al., 2013) was utilized where hESC colonies were manually detached and cultured as floating spheres in Ultra Low Cell Culture Flasks (Corning Inc.) and in media consisting of X-Vivo 15 (Lonza Group Ltd.; Basel, Switzerland), 1X N2, 100ng/ mL bFGF, and 100ng/mL EGF (Life Technologies). Spheres were split approximately every 2 weeks via mechanical trituration using a wide-end P1000 pipette tip with care taken to avoid dissociation to single cells. 5 days prior to adherent monolayer culture, 10ng/mL LIF (EMD Millipore) was added to the sphere culture media (Xeno-Free Neural Stem Media, or XF-NSM). To begin adherent monolayer culture, spheres were plated onto CELLstart coated plates in XF-NSM. Within 1-2 days following sphere attachment, single cells began migrating away from the large sphere and upon reaching 80-90% confluence were dissociated using TrypLE Select (Life Technologies) and replated onto CELLstart coated

plates in XF-NSM. Cells were then split in this manner every 4-6 days. All karyotype analysis of cell lines was performed off-site (Cell Line Genetics Inc.; Madison, WI).

For neural differentiation, TrypLE Select dissociated single cells were plated onto CELLstart coated Lab-Tek Permanox chamber slides (Thermo Fisher Scientific/Nunc) in XF-NSM. 24 hours after attachment, the media was changed to differentiation media (DM) consisting of X-Vivo 15, 10ng/mL BDNF (Peprotech), 10ng/mL GDNF (Peprotech), 1X N2, 1X B27 (Life Technologies), 2ng/mL Heparin (Sigma-Aldrich; St. Louis, MO), 63µg/mL NAC (Sigma-Aldrich), 0.1ng/mL bFGF, and 10µg/mL Ciprofloxacin (Mediatech, Inc.). The media was changed every 3 days with half being removed and replaced with fresh DM. Differentiation was carried out for a total of 2-4 weeks before cells were permeabilized and immunostained.

Magnetic-Activated Cell Sorting and Flow Cytometric Analysis

Magnetic-Activated Cell Sorting (MACS, Miltenyi Biotec) was performed using an autoMACS Pro Separator (Miltenyi Biotec) according to manufacturer-provided protocols via a two-step process: 1) Positive selection of CD133+ cells (retain), followed one passage later by 2) Negative selection for CD34+ cells (remove), to obtain a CD133+/CD34- enriched cell population. Human serum albumin (HSA, Octapharma USA Inc.) was used in place of bovine serum albumin. Magnetic microbead kits human CD133 microbead kit (130-050-801, Miltenyi Biotec) and human CD34 microbead kit (130-046-702, Miltenyi Biotec) were used. All cells were grown prior to sorting as well as post-sorting on CELLstart coated plates in XF-NSM. TrypLE was used to dissociate cells prior to sorting.

For flow cytometric analysis, antibodies used were human CD133/2 (293C3)-PE (130-090-853, Miltenyi Biotec) and human CD34-FITC (130-081-001, Miltenyi Biotec). Surface marker staining was performed according to supplied antibody protocols. Briefly, pelleted cells were resuspended in 80µL MACS buffer. 20µL of FcR Blocking Reagent and 10µL of each respective antibody (either alone, or in combination) were then added to the cell suspension. The suspension was then mixed and incubated at 4C for 10 minutes. Following antibody incubation the cells were washed then incubated with Annexin V according to manufacturer protocols (Life Technologies) for 15 minutes at room temperature. Human IgG beads (BD Biosciences) were used as fluorescent antibody binding controls. All flow cytometry analysis was performed using a BD FACSAria II and FACS Diva and FlowJo (ver. 10.0.6) software.

in vitro Immunocytochemistry

Immunocytochemistry procedures were as described previously (Piltti et al., 2011) with minor modifications. Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized in PBS containing 0.5% Triton-X100 (Sigma-Aldrich) for 20 minutes. The cells were then blocked in PBS containing 5% donkey serum (Jackson ImmunoResearch), and 1% BSA (Sigma-Aldrich) for 10 minutes, followed by primary antibody incubation in blocking buffer for 2 hours at room temperature. Primary antibodies included monoclonal mouse anti-SSEA4 (1:75, ab16287, Abcam), polyclonal rabbit anti-Oct4 (1:200, ab19857, Abcam), polyclonal rabbit anti-Sox2 Alexa Fluor 488-Conjugated (1:100, AB5603A4, EMD Millipore), monoclonal mouse anti-hNestin (1:200, MAB1259, R&D Systems), monoclonal mouse anti-ßIII Tubulin (1:500, MMS-435P,

Covance), polyclonal rabbit anti-GFAP (1:500, Z0334, DakoCytomation), and polyclonal goat anti-hOlig2 (1:100, AF2418, R&D Systems). Following primary antibody incubation and washes, cells were incubated in appropriate secondary antibodies for 1 hour at room temperature and then mounted with Flouromount-G (SouthernBiotech) for imaging. Secondary antibodies included: Alexa Fluor 488 Donkey Anti-Rabbit, Alexa Fluor 555 Donkey Anti-Mouse, Alexa Fluor 488 Donkey Anti-Mouse, Alexa Fluor 568 Donkey Anti-Rabbit, and Alexa Fluor 647 Donkey Anti-Goat. Hoechst 33342 (H1399) was used for nuclear identification. All secondary antibodies and Hoechst were from MolecularProbes/ Life Technologies and used at a 1:1000 dilution. All secondary antibodies were tested for cross-reactivity and non-specific binding. Imaging was performed using an Olympus FluoView FV10i (Olympus America Inc.) and a Zeiss Axio Imager.M2 Apotome System (Carl Zeiss).

RNA Extraction and PCR Analysis

Total RNA was isolated from harvested cells from 2 wells of a 6-well culture plate by scraping with lysis buffer from an RNeasy mini kit (Qiagen). Extracted total RNA was treated with RNase free DNase I (DNA free; Ambion), and synthesis of cDNA was performed by oligo(dT)/random primer mediated reverse transcription with a minimum of 300ng of total RNA input using a high capacity RNA-to-cDNA kit (Applied Biosystems). For quantitative PCR experiments, each reaction was performed using 100ng of cDNA with TaqMan® probe-based gene expression primers that were analyzed per each gene (technical duplicates) in biological triplicate (three independent experiments) for all samples for each cell stage in the study. For non-quantitative PCR experiments, GoTaq

Green Master Mix (Promega Corp.) was combined with specifically designed forward and reverse primers and 200ng cDNA. PCR conditions used were 95°C denaturation, 62°C annealing, and 72°C extension for either 35 or 40 cycles. Due to the number of comparisons, samples of the same cycle number were run in parallel across multiple agarose gels on the same day, and identical exposure settings were used to capture all gel band images. Images were then arranged side-by-side in the figure for ease of visual comparison. All kits were used according to manufacturer's instructions. For primers used see Table S1.

In vitro Cell Growth and Proliferation Analysis

To assess cell growth and obtain mean doubling times of both non-sorted and CD133+/CD34- hNSCs, cells were seeded at an initial density of $5x10^5$ cells per well onto CELLstart-coated 6-well plates. Cells were harvested and dissociated into single cells using TrypLE and counted manually via Trypan Blue exclusion at 24-, 48-, 72-, 96-, and 120-hr post-plating. All cell growth assays were performed in biological triplicates. To assess proliferation via 5-ethynyl-2'-deoxyuridine (EdU, C10337, Life Technologies) incorporation, cells were plated onto CELLstart coated Permanox chamber slides and allowed to grow to approximately 80-90% confluence before adding 10μM EdU and incubating for 24 hours. Post-EdU incubation, cells were fixed and EdU incorporation was detected via a Click-iT® reaction. The quantification of EdU-positive cells to total number of Hoechst-positve nuclei was performed by analyzing fluorescent images of at least 8 randomly chosen fields from 3 independent independent experiments using Volocity 3D Image Analysis Software (PerkinElmer) *(Piltti et al., 2011)*.

Human Stem Cell Preparation for Transplantation

All animal housing conditions, surgical procedures, and postoperative care was approved by and conducted according to the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of California, Irvine. All hESC and hNSC preparations took place during the day of transplantation/injection. All cells were maintained under normal culture conditions prior to harvesting and collection for hippocampal/ subcutaneous injection. For hippocampal hNSC injections, cells were dissociated to single cells using TrypLE and resuspended in X-Vivo 15 to yield $7.5x10^4$ cells/µl. hESCs were harvested in a similar manner for hippocampal injection but care was taken to avoid single-cell dissociation by using a cell scraper (BD Falcon) rather than TrypLE. Hippocampal cell preparations were kept at room temperature.

For subcutaneous leg injections of both hESC and hNSC, cells were prepared similarly to hippocampal preparations but were resuspended in Matrigel rather than X-Vivo 15 and kept on ice to prevent Matrigel from polymerizing prematurely. Dilutions ranged from 1.25 to $2.5x10^6$ cells per 50µl.

Intracranial and Subcutaneous Cell Transplantation

For intracranial injections, both male and female NOD-*scid* mice (12-20 weeks old; The Jackson Laboratory) were anesthetized with 2% isoflurane and positioned in a stereotaxic holder (Leica Microsystems Inc.). A midline incision was made exposing the skull. A 5*µ*l Hamilton syringe (Cat #87930) with a custom 1" 30G blunt needle was mounted into an UMP-3 (World Precision Instruments) injector connected to a SYS-Micro4

Controller. The needle was then moved to 1.94mm posterior to bregma and 2.00mm lateral to midline, and a mark was made using a mechanical pencil. The needle was moved out of the way, and a burr hole was drilled using a Dremel rotary tool at ~5,000 rpm. Cells were first triturated 5 times with a 10 µl pipette then pulled up into the syringe over approximately 10 seconds. The needle was first lowered to 2.15mm ventral relative to bregma to create a pocket in the brain, then raised to 2.10mm. Cells were then injected at doses ranging from $7.5x10^4$ to $1.5x10^5$ (at $7.5x10^4$ cells/µl) over 2 minutes. Speeds varied relative to total volume, but time of injection remained constant. The needle was raised out of the brain 4 minutes after injection. The syringe was tested to make sure it was not clogged by ejecting some of the solution into the air manually through the Micro4 controller. Bone wax was applied to seal the burr hole, and the midline incision was stapled shut.

For subcutaneous injections, anesthetized mice received 50µl injections of either cells (hESC or hNSC) or vehicle control (Matrigel, BD Biosciences) via injection through a 23G needle attached to a 1ml syringe that was inserted underneath the skin, between the knee and ankle. The needle was held in place for approximately 10 seconds before retraction, and all cell/vehicle preparations, and injection equipment, were kept on ice to prevent Matrigel from polymerizing prematurely.

Animals were randomly allocated to different cell type and injection location groups as described in Table 1 and were furthermore processed blind to injection cohort. Animals received lactated ringers (50 ml/kg) subcutaneously immediately after surgery as well as Buprenorphine (0.5 mg/kg) immediately after surgery and for 2 days thereafter. Additionally, due to the immunodeficient nature of NOD-*scid* mice, an antibiotic (Baytril, 2.5

mg/kg) was administered immediately after surgery and daily for 5 days thereafter. No immunosuppressant drugs were administered to any animal.

Tissue Collection and Immunohistochemistry

Tissue collection and immunohistochemistry was performed as previously described (Hooshmand et al., 2009) with minor modifications. Briefly, all animals, ranging from 8-20 weeks post-transplant, were anesthetized with a lethal dose of Euthasol (100 mg/kg, i.p.) and transcardially perfused with 30 ml of PBS, followed by 100 ml of 4% paraformaldehyde. Brains were carefully dissected and post-fixed overnight in a solution of 4% paraformaldehyde and 20% sucrose in PBS at 4°C, flash frozen at −65°C in isopentane (2-methyl butane), and stored at −80°C.

For cryosectioning and immunohistochemistry, frozen brains were embedded in Optimal Cutting temperature (O.C.T.) compound (Sakura Finetek USA, Inc.). 35µm thick coronal sections were cut on a sliding microtome, collected in 96-well plates containing 0.1M Tris and 0.02% Sodium Azide, and kept at 4°C until processed for immunostaining. All immunostaining procedures were conducted at room temperature. Briefly, sections were washed in 0.1M Tris followed by a 15 minute incubation in 0.3% hydrogen peroxide/ methanol. After a brief 0.1% TritonX-100 wash, sections were blocked for 1 hour with bovine serum albumin (BSA) and normal serum from the species in which the secondary antibody was raised. Sections were then exposed overnight to the appropriate primary antibody. The primary antibodies used were: monoclonal mouse anti-SC121 (1:10,000), monoclonal mouse anti-SC101 (1:2,000), mouse monoclonal anti-SC123 (1:3,000) (all from StemCells Inc.), monoclonal mouse anti-hNestin (1:1,000) (MAB1259, R&D Systems),

polyclonal rabbit anti-PDX1 (1:2,000) (ab47267, Abcam), polyclonal rabbit anti-αSMA (1:100) (ab5694, Abcam), monoclonal rabbit anti-ßIII Tubulin (1:500) (MRB-435P, Covance), polyclonal rabbit anti-VGlut1 (1:50, ab72311, Abcam), and polyclonal rabbit anti-GAD65+67 (1:1000, ab49832, Abcam). The next day, sections were incubated with either fluorescent or biotin-conjugated, purified IgG secondary antibody (1:500, Jackson Immunoresearch) pre-adsorbed against the species in which the primary was raised, followed by avidin-biotinylated peroxidase complex (ABC) using a Vectastain Elite ABC kit (Vector Laboratories, USA) prepared according to the manufacturer's recommendations (for DAB staining). After several washes, the signal was visualized with diaminobenzidine (DAB) (Vector Laboratories, USA). Sections were mounted onto slides and allowed to dry overnight at 37°C. DAB stained sections were lightly counterstained with the nuclear marker, methyl green. Fluorescent stained sections were counterstained with Hoechst 33342 (1:1000, Life Technologies). All slides were coverslipped using Depex or Fluoromount-G (SouthernBiotech) mounting medium. Imaging was performed using an Olympus FluoView FV10i (Olympus America Inc.) and also a Zeiss Imager.M2 Apotome System (Carl Zeiss).

Statistical Analysis

All qPCR and EdU/growth kinetics data are representative of three or more individual independent experiments. Grubb's test (ESD method) was used to eliminate qPCR replicate statistical outliers. Errors are the standard error (SEM) of averaged results. EdU data analyzed by one-way ANOVA (Bonferroni post-hoc) with $P < 0.001$ using GraphPad Prism software.

Results

Xeno-Free Transition and Neuralization of Three Human Embryonic Stem Cell Lines

Culture of the human embryonic stem cell lines Shef3, Shef4, and Shef6 was initially established in "standard" conditions that contain both bovine and murine components (Figure 2.1B-D, Non-XF hESC). To enhance the clinical applicability of these human stem cells lines, all hESC lines were transitioned to "Xeno-Free" conditions in a 1-step simultaneous conversion of both media (to a human serum-based media) and substrate (to a human vitronectin-based substrate, CELLstart) (Figure 2.1A). XF transitioned hESCs typically required an adaptation or stabilization period of 1-2 passages post-transition in which spontaneously differentiating cells were removed via manual dissection. Following XF transition, all three XF hESC lines maintained circular, hESC-like colony morphologies and expressed the pluripotency markers Oct4 and SSEA4 at both 9-11 passages in XF conditions (Figure 2.1B-D, XF hESC) as well as after prolonged XF hESC culture (Figure S2.1D).

After 9 to 11 passages in XF hESC conditions (40-60 days in culture), transitioned XF hESCs were subsequently subjected to neural induction via EGF/FGF supplementation to generate intermediate neuralized spheres (Ebert et al., 2013). Further neuralization of spheres with LIF supplementation and cell attachment on CELLstart generated an adherent monolayer culture system (Figure 2.1A) that produced cells with bi- and multi-polar neural morphologies (Figure 1B-D, XF hNSC). One cell line (Shef3) was determined to be karyotypically abnormal after XF transition and neuralization (Figure 2.1B, karyotype; Figure S2.1). The other two hNSC lines (Shef4, Shef6) were karyotypically normal (Figure

2.1C-D, karyotype). Prior to any subsequent *in vitro* or *in vivo* studies, XF adherent hNSCs had, at a minimum, been cultured in XF conditions for over 100 days (40-60 days as XF hESC, followed by 60+ days as XF hNSC spheres/monolayer), a time period sufficient to eliminate detectable non-human molecules (Ludwig et al., 2006; Heiskanen et al., 2007)

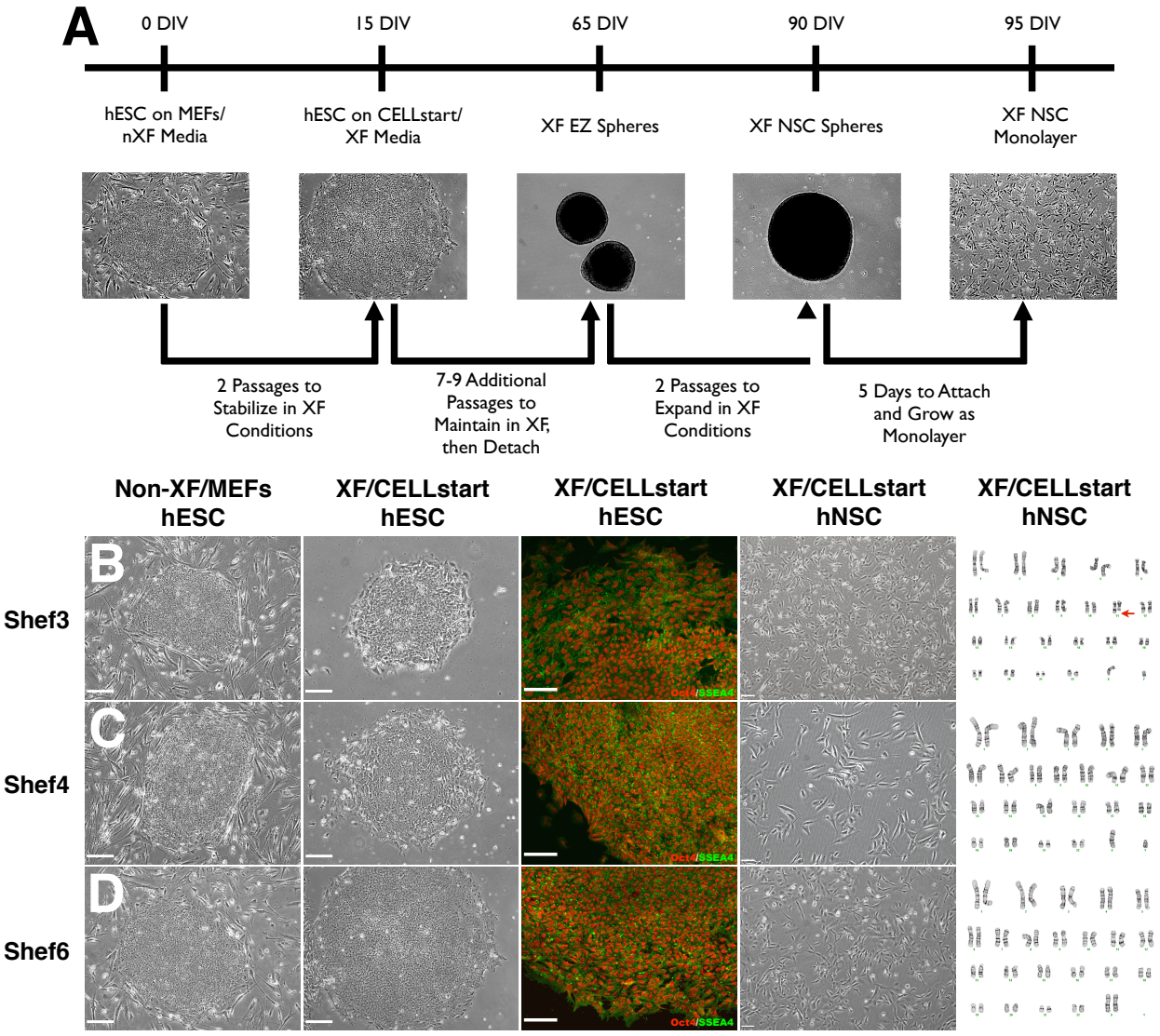

Figure 2.1. hESCs transitioned to XF conditions maintain pluripotency and can be further neuralized to form XF hNSCs

(A) Schematic for the XF transition and subsequent neuralization of hESCs into bi- and multi-polar neural stem/progenitor-like cells via a modified EZ sphere-based protocol.

(B-D) Shef3 (B) undifferentiated hESCs were originally maintained on MEFs and in standard non-XF hESC culture media containing BSA. hESCs were then transitioned to XF conditions

utilizing the XF substrate CELLstart and KnockOut SR XF/KO SR GF Cocktail. Following XF transition, hESCs continued to express the pluripotency markers Oct4 (red) and SSEA4 (green). Finally, XF transitioned hESCs were further neuralized to form adherent XF hNSCs that exhibited characteristic neural morphologies. Subsequent karyotype analysis revealed one chromosomal abnormality (Shef3, Chromosome 11) and two karyotypically normal (Shef4, Shef6) hNSC lines. (C) Shef4 and (D) Shef6 hESCs were similarly transitioned to XF conditions and neuralized. Scale: 200μm (hESCs, phase), 100μm (hESCs, fluorescent) and 50μm (hNSCs). See also Figure S2.1.

Magnetically Sorted (CD133+/CD34-) Xeno-Free Shef6 hNSCs Exhibit Increased Neuronal Differentiation in vitro

Following XF neuralization, magnetic sorting was used to further enrich one of the hNSC lines, Shef6, for a stem/progenitor population via positive selection of hNSCs expressing CD133 and depletion of cells expressing CD34. Flow cytometric analysis of non-sorted XF Shef6 hNSCs demonstrated that ~17% expressed CD133, and less than 2% expressed CD34 (Figure 2.2A). Post-sorting, the CD133+ proportion increased to ~82%, while CD34 remained at less than 2% (Figure 2.2B). The increase in the CD133+ proportion of cells was maintained over long-term culture as greater than 70% of sorted XF Shef6 hNSCs still expressed CD133 after 20 additional passages (P27, Figure 2.2C).

Interestingly, while both XF undifferentiated non-sorted and CD133+/CD34- sorted Shef6 hNSCs expressed the neural stem and progenitor markers Sox2 and Nestin (Figure 2.2D-E), morphological differences were observed between the two neuralized populations. Notably, non-sorted cells appeared larger and more cytoplasmic (Figure 2.2D) than sorted cells (Figure 2.2E). Additionally, analysis via qPCR (Figure 2.2H) demonstrated a reduction of pluripotent (Oct4, Nanog) and embryonic germ layer (Brachyury, Desmin, GATA-4, Sox17) mRNA expression in the neuralized populations compared to XF Shef6 hESCs. Furthermore, the sorted XF Shef6 hNSCs also expressed higher levels of neural lineage

markers Nestin (6-fold upregulation in P7 CD133+/CD34- sorted hNSCs, 12.5-fold in P27 sorted hNSCs vs. non-sorted Shef6 hNSCs) and Pax6 (115-fold upregulation in P7 CD133+/ CD34- sorted hNSCs, 600-fold in P27 sorted hNSCs) as well as smaller increases in both CD133 and Ki67 expression and a decrease in CD34 expression in comparison to non-sorted XF Shef6 hNSCs.

Differences were also observed between the two populations in cells undergoing neural differentiation, with non-sorted cells expressing neuronal Class III ß-Tubulin (ßIII-Tub) and GFAP in elongated, bipolar cells (Figure 2.2F) while CD133+/CD34- cells expressed primarily ßIII-Tub in densely populated areas (Figure 2.2G) as well as GFAP in sparse but clustered regions (Figure S2.1C). Morphologically, in the sorted population, ßIII-Tub immunostaining was observed in numerous spineous bipolar and multipolar neuronal cells and GFAP in larger, flatter astrocytic cell types. qPCR was used to further probe the differentiation potential of the two populations. As observed via ICC, there was an increase in neuronal differentiation (ßIII-Tub 7-fold upregulation, Dcx 89-fold, Synapsin 22-fold) in the sorted XF Shef6 hNSCs (P7) in comparison to non-sorted hNSCs (Figure 2.2I). Interestingly, sorted XF Shef6 hNSCs also exhibited a decrease in glial fate potential (PDGF-Ra, GalC, CSPG, GFAP) compared to non-sorted cells, suggesting that CD133+ enrichment at this developmental stage may bias cells towards a neuronal fate. Importantly, even though the sorted populations exhibited enhanced proliferation as detected by Ki67 gene expression under non-differentiating conditions in comparison to non-sorted XF Shef6 hNSCs (Figure 2.2H), there do not appear to be significant differences once the hNSCs have undergone differentiation (Figure 2.2I), suggesting a downregulation of proliferative potential upon differentiation.

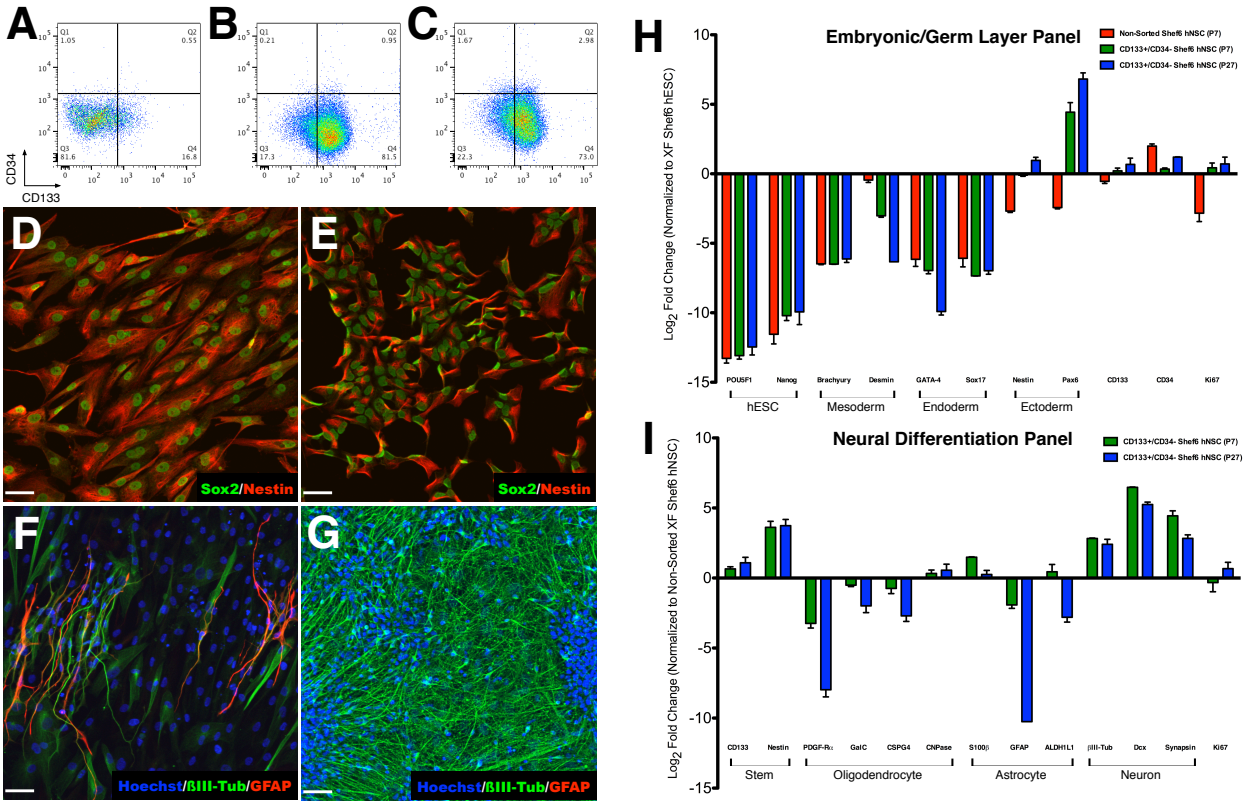

Figure 2.2. Undifferentiated CD133+/CD34- sorted XF Shef6 hNSCs have reduced levels of embryonic/germ layer expression, and generate fewer glial cells but more neurons after *in vitro* differentiation

(A-C) Flow cytometric analysis of (A) non-sorted (P7), (B) low passage (P7) CD133+/CD34- sorted, and (C) high passage (P27) CD133+/CD34- sorted XF Shef6 hNSCs displayed an increase in CD133+ proportion post-sorting (16.8% to 81.5%, non-sorted to low passage sorted) that was maintained at higher passages (73.0%, high passage sorted). The CD34+ proportion remained under 5% for all samples.

(D-G) Immunocytochemistry of both (D) non-sorted and (E) low passage CD133+/CD34- sorted XF Shef6 hNSCs expressed neural stem/progenitor markers Sox2 (D-E, green) and Nestin (D-E, red) *in vitro* under non-differentiating conditions. Upon differentiation, both (F) non-sorted and (G) low passage CD133+/CD34- sorted XF Shef6 hNSCs expressed more mature neural lineage markers ßIII-Tub (F-G, green) and GFAP (F, red; Figure S1C, red). Nuclei were counter-stained with Hoechst 33342 (F-G, blue). Scale: 50µm (D-G).

(H-I) qPCR analysis of (H) embryonic and germ layer/early differentiation markers expressed in both non-sorted and CD133+/CD34- sorted undifferentiated XF Shef6 hNSCs normalized to XF Shef6 hESCs and (I) neural lineage differentiation markers expressed in CD133+/CD34- sorted XF Shef6 hNSCs after 28DIV neural differentiation normalized to non-sorted XF Shef6 hNSCs. All data were normalized to internal 18s rRNA and expressed as mean log2 fold change ± SEM (n = 3 independent experiments with technical duplicates of each independent biological experiment).

Furthermore, regional identity of both the non-sorted and CD133+/CD34- sorted Shef6 hNSCs was evaluated via RT-PCR (Figure 2.3A-B). Non-sorted Shef6 hNSCs expressed hindbrain markers GBX2 and KROX20, and lacked forebrain and forebrain/midbrain marker expression (FOXG1and OTX2, respectively). Non-sorted Shef6 hNSCs also lacked expression or expressed very low levels of the neural stem cell markers PAX6 and ASCL1 as well as neural rosette markers PLZF and DACH1. Similarly, both low passage (P7) and high passage (P27) CD133+/CD34- sorted Shef6 hNSCs expressed the hindbrain markers GBX2 and KROX20, and also lacked or had very low expression of forebrain markers FOXG1 and OTX2. However, the sorted hNSCs exhibited expression of both neural stem (Pax6, ASCL1) and rosette-specific (PLZF, DACH1) markers, suggesting that the CD133+/CD34- Shef6 hNSCs are most likely an expanding neural stem-like cell population possessing a hindbrain regional specification preference.

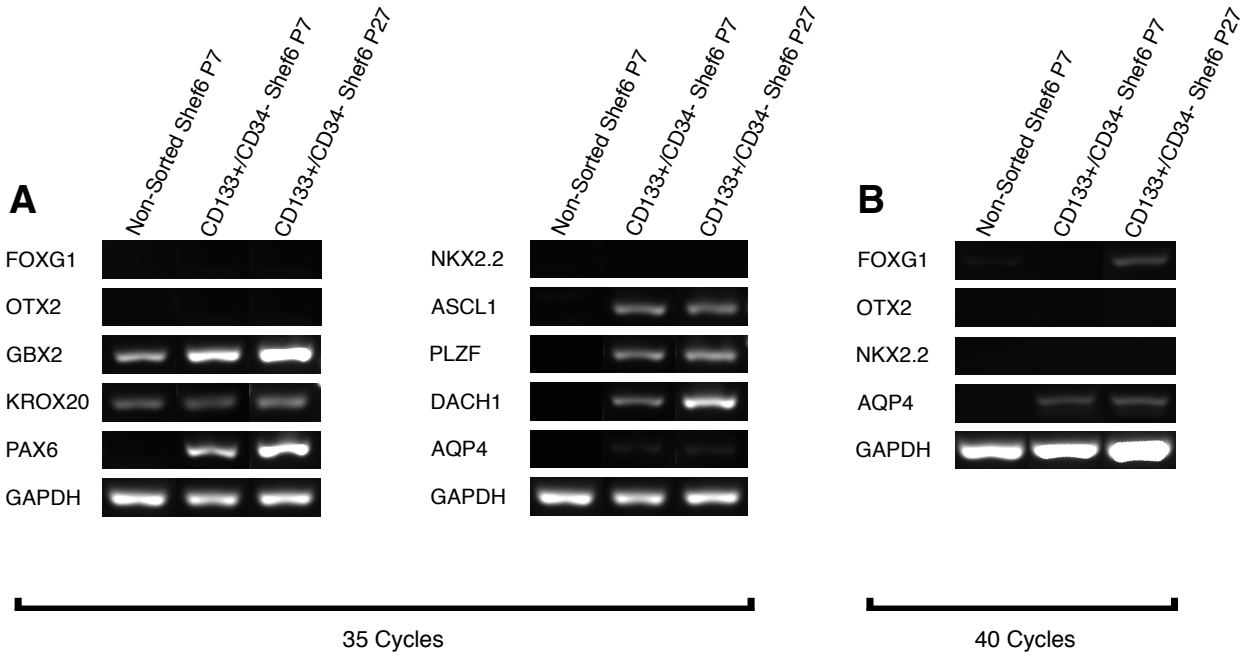

Figure 2.3. CD133+/CD34- sorted Shef6 hNSCs express hindbrain as well as neural stem and rosette-specific transcription factors *in vitro*
(A-B) RT-PCR analysis of non-sorted XF Shef6 (P7), low passage (P7) CD133+/CD34- sorted, and high passage (P27) CD133+/CD34- sorted XF Shef6 hNSCs at (A) 35 cycles of PCR amplification exhibited expression of hindbrain transcription factors GBX2 and KROX20, and lacked expression of forebrain and forebrain/midbrain factors FOXG1and OTX2, respectively, as well as the ventral marker NKX2.2 and the more mature FGF/EGF-expanded marker AQP4. CD133+/CD34- sorted Shef6 hNSCs demonstrated expression of neural stem cell markers PAX6 and ASCL1, as well as neural rosette markers PLZF and DACH1, whereas non-sorted Shef6 hNSCs exhibited very low to no expression of these neural stem/rosette markers. (B) At 40 cycles of PCR amplification, CD133+/CD34- sorted Shef6 hNSCs exhibited very low levels of FOXG1 (P27 hNSCs) and AQP4 expression (P7 and P27), but no or extremely low expression of OTX2 and NKX2.2.

Magnetically Sorted (CD133+/CD34-) Xeno-Free Shef6 hNSCs Proliferate Extensively in vitro

In addition to differences in morphology and differentiation propensity, differences in proliferation and cellular growth kinetics were also observed between non-sorted and sorted XF Shef6 hNSCs. EdU incorporation over a 24-hour period was used to gauge proliferation of undifferentiated hNSCs, with 21% of non-sorted XF Shef6 hNSCs (Figure 2.4A, C) incorporating EdU compared to 91% of CD133+/CD34- sorted XF Shef6 hNSCs (Figure 2.4B, C). EdU incorporation remained consistent after 20 additional passages of prolonged culture (95%, Figure 2.4C). Additionally, non-sorted Shef6 hNSCs expanded to ~850,000 cells from an initial starting density of 500,000 cells over the course of 5 days *in vitro* (Figure 2.4D), giving a doubling time of 5.1-6.4 days. Conversely, over the same 5 day period, CD133+/CD34- sorted Shef6 hNSCs expanded to over 14 million cells (Figure 2.4D) from the same initial density of 500,000 cells (a doubling time of 0.9-1.4 days). The overall increase in cell expansion of sorted XF Shef6 hNSCs in comparison to non-sorted XF Shef6 hNSCs was maintained after 20 additional passages (Figure 2.4D), although a minor decrease in expansion capability was observed between low (P7, 0.9-1.4 days doubling)

and high passage (P27, 1.0-1.4 day doubling) sorted hNSCs. Interestingly, non-sorted and CD133+/CD34- sorted Shef4 hNSCs exhibited similar *in vitro* expansion rates to each other, but both were slower than the non-sorted Shef6 hNSCs (data not shown). Due to the slow proliferative nature of the Shef4 hNSCs, as well as the karyotypic abnormality in the Shef3 hNSCs, further *in vivo* studies utilized Shef6 hNSCs exclusively.

Taken together, the EdU and growth kinetics data suggest a dramatic increase in proliferative potential of the sorted XF Shef6 hNSCs *in vitro* such that a starting population of 2 million hNSCs could be expanded to over 300 million hNSCs in ~10 days (Figure 2.4E).

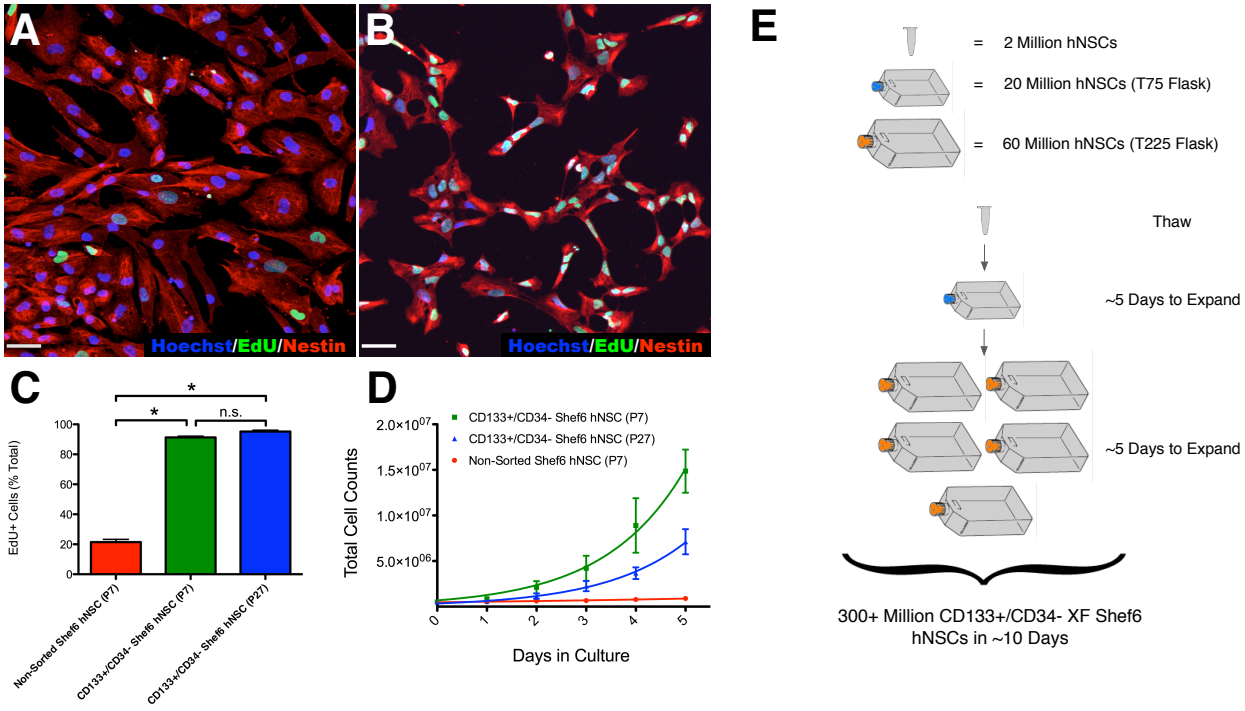

Figure 2.4. CD133+/CD34- XF Shef6 hNSCs proliferate extensively *in vitro*
(A-B) Undifferentiated (A) non-sorted (P7) and (B) low passage (P7) CD133+/CD34- sorted XF Shef6 hNSCs incorporated EdU over a 24-hour pulse period. EdU (A-B, green) and Nestin (A-B, red). Nuclei were counterstained with Hoechst 33342 (A-B, blue). Scale: 50µm (A-B).
(C) Quantification of EdU incorporation as a percentage of total cells (EdU+Hoechst+/Hoechst +). Error bars are mean percentage ± SEM (n = 3 independent independent experiments) analyzed by ANOVA (Bonferroni), * $P < 0.001$.
(D) Growth kinetics of non-sorted (P7) as well as both low (P7) and high passage (P27) CD133+/CD34- sorted XF Shef6 hNSCs plated on CELLstart at an initial density of 500,000

cells and cultured for 5 subsequent days. Cells were counted daily at the same time. Error bars are mean cell counts ± SEM (n = 3 independent experiments).
(E) Schematic example of CD133+/CD34- sorted XF Shef6 hNSC expansion capability to generate in excess of 300 million hNSCs from one thawed vial (containing 2 million hNSCs) over the course of ~10 days.

Xeno-Free CD133+/CD34- Sorted hNSCs Do Not Form Teratomas in vivo and Differentiate Primarily into Neurons

To examine tumorigenicity potential, intrahippocampal transplanted non-sorted and CD133+/CD34- sorted Shef6 hNSCs were compared to similarly transplanted undifferentiated Shef6 hESCs. As expected from a pluripotent cell population, transplanted Shef6 hESCs exhibited graft overgrowth with widespread intrahippocampal migration, that in many cases caused the endogenous tissue architecture to become deformed (Figure 2.5A). Transplanted Shef6 hESCs also expressed both neural and non-neural (Figure 2.5B-D) germ layer-specific markers, including hNestin (ectoderm), α-SMA (mesoderm), and PDX1 (endoderm) at 8 weeks post-transplantation.

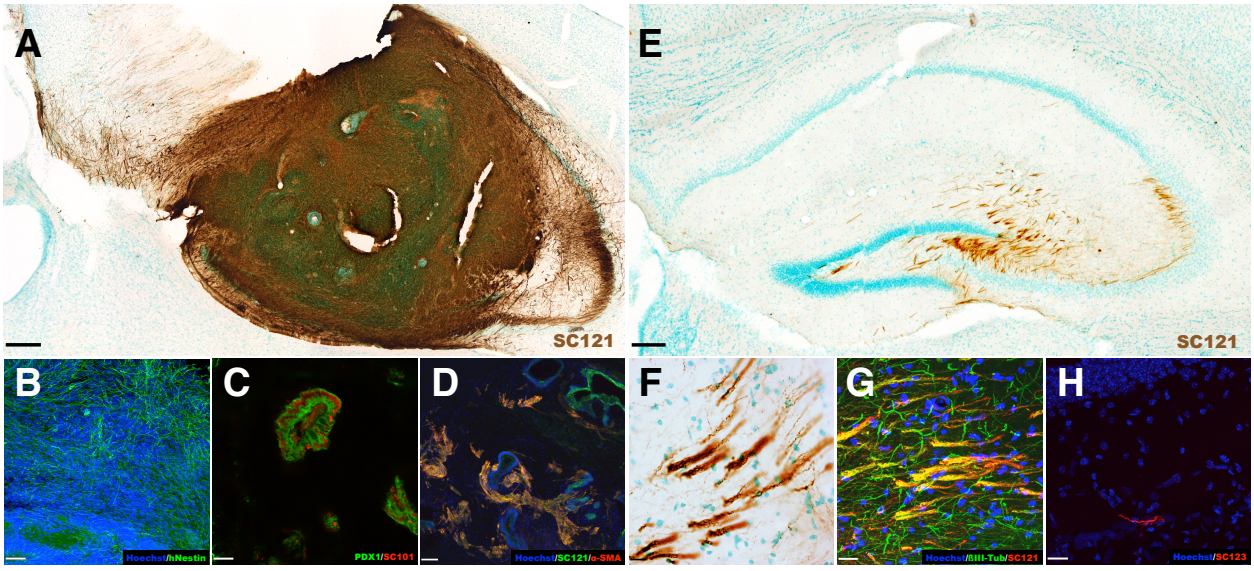

Figure 2.5. Evaluation of tumorigenesis potential - *in vivo* engraftment and fate of hippocampal transplanted XF Shef6 hESC and CD133+/CD34- sorted XF hNSCs

(A-D) Transplanted XF Shef6 hESCs (Dose: 75,000 cells) survive and engraft in the immunodeficient NOD-*scid* hippocampus at 8-weeks post-transplantation. (A) Formation of large, dense human-positive (SC121, brown) masses that engulf the Shef6 hESC transplanted hippocampus. Within the transplanted region, cells of each embryonic germ layer lineage were detected, including (B) ectoderm (hNestin, green), (C) endoderm (PDX1, green; SC101, red), and (D) mesoderm (α-SMA, red; SC121, green). Scale: 200µm (A), 50µm (B, C), 80µm (D). (E-H) Transplanted low passage (P7) CD133+/CD34- sorted XF Shef6 hNSCs (Dose: 150,000 cells) survive and engraft in the immunodeficient NOD-*scid* hippocampus at 12-weeks post-transplantation. (E) Engraftment of Shef6 hNSCs (SC121, brown) and migration into the molecular layer as well as dentate hilar and CA3 hippocampal regions. (F) Transplanted Shef6 hNSCs often form fibrous bundles that (G) stain positive for the neuronal marker ßIII-Tubulin (ßIII-Tub, green; SC121, red). (H) Human astrocytes (SC123, red) were also visualized occasionally. For (A, E, and F) methyl green was used to counterstain nuclei. For (B, D, G, and H) Hoechst 33342 was used to counterstain nuclei (blue). Scale: 200µm (E), 20µm (F-H). See also Figure S2.2.

Additionally, subcutaneous leg injections of both Shef6 hESC and hNSC populations were performed at doses ranging from 1.25 million to 2.5 million cells per injection. Only the Shef6 hESC population displayed gross anatomical abnormalities, with 79% of injected animals exhibiting large masses (Table 2.1, Figure S2.2).

Table 2.1. Engraftment and tumorigenesis potential of XF Shef6 hESCs and hNSCs

Cell Line/ Type	Leg Injection Dose	# Leg Grafts	% with Leg Tumors	CNS Injection Dose	# CNS Grafts	% with CNS Engraftment	% Engrafted with CNS Tumors
Shef6 hESC	1,250,000	14	79% (11/14)	75,000	8	63% (5/8)	100% (5/5)
Shef6 hNSC (non-sorted)	1,250,000	10	0% (0/10)	75,000	8	0% (0/8)	No Engraftment
Shef6 hNSC (non-sorted)				150,000	2	0% (0/2)	No Engraftment
Shef6 hNSC (sorted)	1,250,000	23	0% (0/23)	75,000	15	93% (14/15)	0% (0/14)
Shef6 hNSC (sorted)	2,500,000	17	0% (0/17)	150,000	4	100% (4/4)	0% (0/4)

Table 2.1. Engraftment and tumor forming potential of subcutaneously (leg) and intracranially (hippocampus) injected XF Shef6 hESCs as well as both non-sorted and low passage (P7) CD133+/CD34- sorted hNSCs at multiple doses. Shef6 hESC transplants resulted in tumors in 79% (leg) and 63% (CNS) of transplants at 8-12 weeks post-transplant. All animals with successful CNS hESC engraftment exhibited tumors. No non-sorted hNSCs were detected 12

weeks post-transplant. CD133+/CD34- sorted Shef6 hNSCs engrafted in the CNS at a rate of 93-100% and no CD133+/CD34- sorted Shef6 hNSC transplants resulted in tumors. See also Figure S2.2.

Conversely, intrahippocampal transplanted Shef6 hNSCs (as opposed to Shef6 hESCs) exhibited markedly different engraftment dynamics. We saw no evidence of cell engraftment in any of the 10 animals transplanted with non-sorted Shef6 hNSCs (Table 2.1) at 12 weeks post-transplantation. Conversely, a high level of engraftment was observed in CD133+/CD34- Shef6 hNSC transplanted animals (18/19, 95%; Table 2.1). Engrafted cells migrated laterally from the single injection site, spanning from the dentate gyrus to CA3 (Figure 2.5E). The sorted Shef6 hNSCs extended cell projections that often displayed bundled or fasciculated phenotypes (Figure 2.5F). Occasionally, cells appeared to migrate out from hippocampus and were located adjacent to the walls of the lateral ventricle. Engrafted CD133+/CD34- Shef6 hNSCs exhibited primarily neuronal differentiation at 12 weeks post-transplantation, determined via co-expression of the human-specific cytoplasmic marker SC121 and ßIII-Tub (Figure 2.5G). Rare astroglial differentiation was also observed (SC123; Figure 2.5H).

Additionally, SC121+ human cells were nearly always found to be associated with vesicular glutamate transporter 1 (VGlut1) immunopositive neurons (Figure 2.6A-D), suggesting that the majority of the transplanted CD133+/CD34- Shef6 hNSCs differentiated into glutamatergic neurons *in vivo*. Glutamic acid decarboxylase (GAD67) positive puncta were also occasionally associated with SC121+ human cells, although the pattern of GAD67 immunoreactivity appeared more isolated, sparse, and on the surface of SC121+ cells (Figure 2.6E-H), suggesting that the human, glutamatergic neurons were receiving GABAergic innervation rather than being GABAergic themselves. In no cases examined was

non-neural cell type staining found to be co-localized with transplanted human hNSCs (e.g. α-SMA, PDX1)

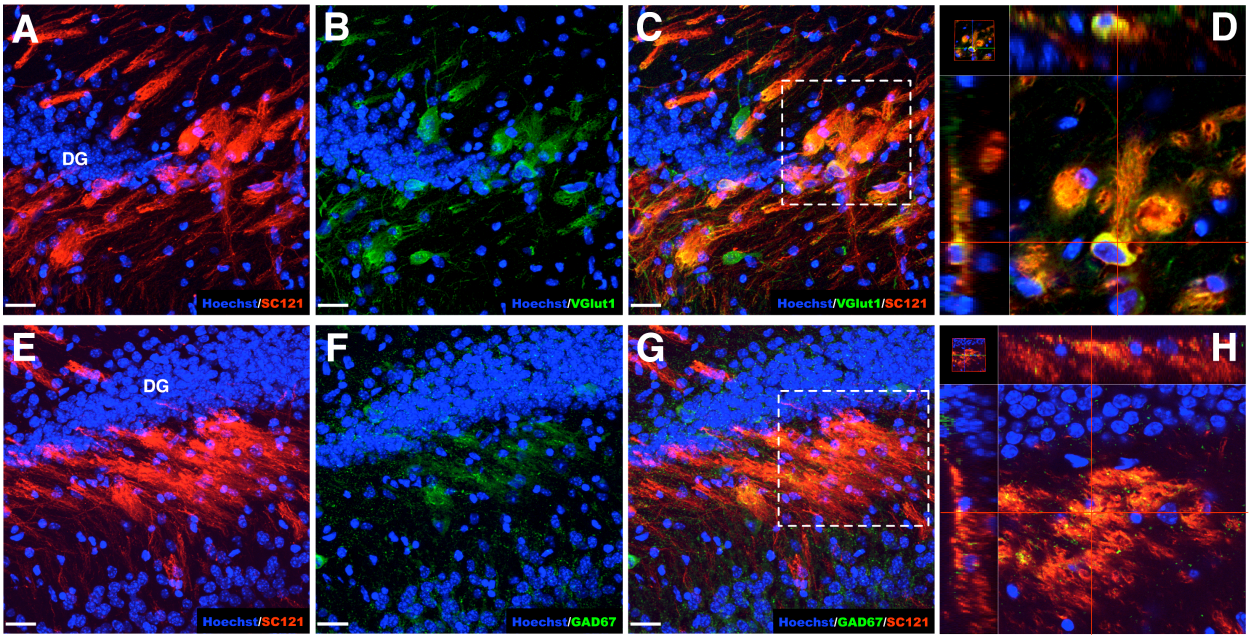

Figure 2.6. Hippocampal transplanted CD133+/CD34- sorted Shef6 hNSCs predominantly differentiate into glutamatergic neurons and receive GABAergic inputs
(A-D) Nearly all engrafted CD133+/CD34- Shef6 hNSCs (A, SC121, red) were immunopositive for vesicular glutamate transporter 1, a glutamatergic neuron marker (B, VGlut1, green), throughout the cytoplasm of the transplanted cells (C, co-expression; D, magnified inset from C). (E-H) A subset of engrafted CD133+/CD34- Shef6 hNSCs (E, SC121, red) also appeared to receive glutamic acid decarboxylase (GAD) positive GABAergic inputs (F, GAD67, green), visualized as small GAD67 immunopositive puncta contacting regions of human SC121+ cytoplasm (G, co-expression; H, magnified inset from G). Hoechst 33342 was used to counterstain nuclei (blue). Scale: 20µm.

Supplemental Information

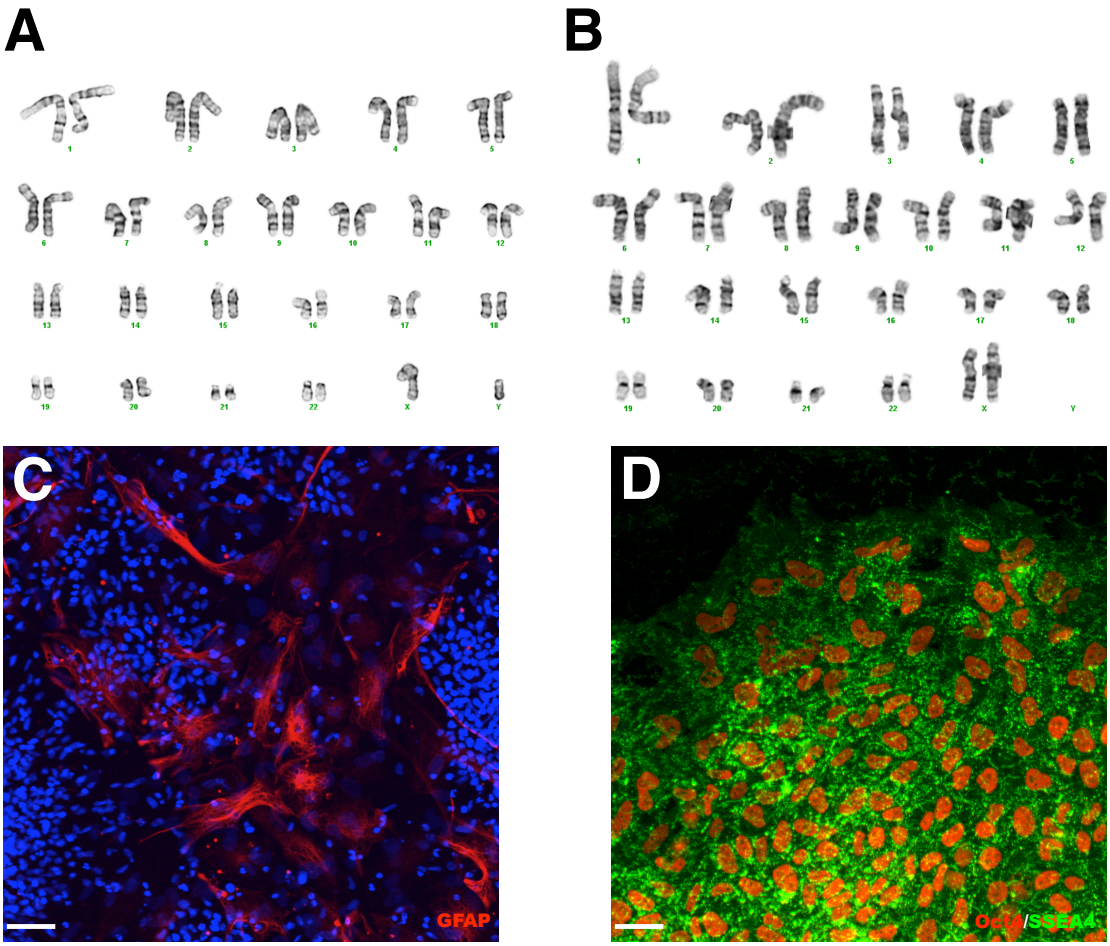

Figure S2.1. Additional karyotype and *in vitro* fate analysis (see also Figures 2.1, 2.2)
(A-B) No karyotypic abnormalities were found in either (A) XF Shef3 hESCs or (B) high passage (P27) CD133+/CD34- sorted XF Shef6 hNSCs.
(C) Immunocytochemistry of low passage (P7) CD133+/CD34- sorted XF Shef6 hNSCs occasionally expressed the astrocyte marker GFAP (red) in small clusters *in vitro* under differentiating conditions. Nuclei were counter-stained with Hoechst 33342 (blue).
(D) Immunocytochemistry of XF Shef4 hESCs after prolonged (27 passages) culture in XF cell culture conditions continued to express the pluripotency markers Oct4 (red) and SSEA4 (green). Scale: 50μm (C) and 25μm (D).

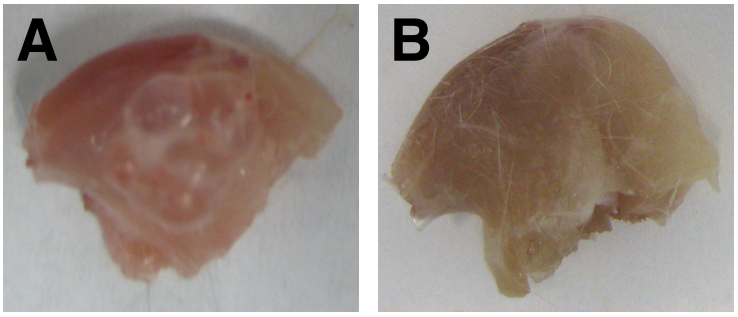

Figure S2.2. XF Shef6 hESCs, but not hNSCs, form subcutaneous (leg) tumors (see also Figure 2.5, Table 2.1)
(A-B) Example of a (A) XF Shef6 hESC-injected gastrocnemius muscle 12-weeks post injection (1.25 million hESCs injected subcutaneously). (B) Example of a low passage (P7) CD133+/CD34- sorted XF Shef6 hNSC-injected muscle 12-weeks post injection (2.5 million hNSCs injected subcutaneously).

Table S2.1. PCR primers (TaqMan and RT-PCR) used in neural derivation and differentiation studies (see also Figures 2.2, 2.3)

Gene	TaqMan ID
18S	Hs99999901_s1
TBP	Hs99999910_m1
POU5F1 (OCT4)	Hs00999632_g1
NANOG	Hs02387400_g1
Brachyury	Hs00610080_m1
Desmin	Hs00157258_m1
GATA-4	Hs00171403_m1
SOX17	Hs00751752_s1
Nestin	Hs00707120_s1
PAX6	Hs00240871_m1
CD133	Hs01009250_m1
CD34	Hs00990734_g1
PDGF-Ra	Hs00998018_m1
GalC	Hs01012300_m1
NG2 (CSPG4)	Hs00361541_g1
CNPase	Hs00263981_m1
S100-B	Hs00902901_m1
GFAP	Hs00909236_m1
ALDH1L1	Hs00201836_m1
ß-Tubulin III	Hs00964962_g1
DCX	Hs00167057_m1
Synapsin	Hs00199577_m1
Ki67	Hs01032443_m1

Gene	Sequence (5' to 3')
GAPDH-F	AAGGTGAAGGTCGGAGTCAAC
GAPDH-R	GGGGTCATTGATGGCAACAATA
FOXG1-F	CCCTCCCATTTCTGTACGTTT
FOXG1-R	CTGGCGGCTCTTAGAGAT
OTX2-F	TGCAGGGGTTCTTCTGTGAT
OTX2-R	AGGGTCAGAGCAATTGACCA
GBX2-F	CTCGCTGCTCGCCTTCTC
GBX2-R	GCCAGTCAGATTGTCATCCG
KROX20-F	TTGACCAGATGAACGGAGTG
KROX20-R	CAGAGACGGGAGCAAAGC
PAX6-F	AATAACCTGCCTATGCAACCC
PAX6-R	AACTTGAACTGGAACTGACACAC
NKX2.2-F	TGCCTCTCCTTCTGAACCTTGG
NKX2.2-R	GCGAAATCTGCCACCAGTTG
ASCL1-F	CGGCCAACAAGAAGATGAGT
ASCL1-R	TGGAGTAGTTGGGGGAGATG
PLZF-F	CTATGGGCGAGAGGAGAGTG
PLZF-R	TCAATACAGCGTCAGCCTTG
DACH1-F	GTGGAAAACACCCCTCAGAA
DACH1-R	CTTGTTCCACATTGCACACC
AQP4-F	GGAATTTCTGGCCATGCTTA
AQP4-R	AGACTTGGCGATGCTGATCT

Discussion

The data presented here establish protocols to transition and maintain multiple hESC and hNSC lines long-term in completely XF culture conditions, thus providing straight-forward methodologies to produce clinically compliant cell lines. Additionally, we have used magnetic sorting to enrich these XF Shef6 hNSCs for cells expressing the surface marker CD133. Sorting for CD133+ cells dramatically increased both the neuronal fate preference as well as the proliferative potential of the Shef6 hNSCs, to the extent that enough cells could potentially be generated within ~10 days for a theoretical human brain transplant (Gupta et al., 2012). Importantly, we have also addressed safety concerns inherent with embryonic stem cell-derived hNSCs. We have demonstrated that while these cells expand rapidly in culture, once they are transplanted into the brain there was no evidence of non-neural lineage tumors. Both XF human cell types (embryonic and neural) generated via the methods presented here exhibited good stability. After more than 10 passages in completely XF conditions, XF hESCs remained karyotypically normal, expressed key pluripotency markers, exhibited minimal spontaneous differentiation, and were able to generate teratomas upon transplantation into NOD-*scid* mice. XF transitioned hESCs were subsequently able to be differentiated into hNSCs in completely XF conditions as well. CD133+/CD34- XF Shef6 hNSCs were able to be maintained for over 25 passages in XF neural conditions, maintained a normal karyotype, exhibited similar differentiation and proliferation profiles to earlier passage hNSCs, and engrafted after intrahippocampal transplantation.

Positive selection via magnetic sorting for CD133 increased the CD133+ proportion of the XF Shef6 hNSCs from 17% to 82%. Interestingly, the CD34+ proportion of the Shef6 hNSCs was measured at less than 5%, as detected by flow cytometry, in both the non-sorted

and CD133+/CD34- sorted hNSCs, which may obviate the need for CD34 depletion in the cell populations discussed herein. However, CD34 is expressed on multiple cell types, including hematopoetic stem cells (Civin et al., 1984; Baum et al., 1992; Goodell et al., 1997), endothelial cells (Pusztaszeri et al., 2006), macrophages (Ryncarz and Anasetti, 1998), and microglia (Asheuer et al., 2004; Ladeby et al., 2005), and therefore warrants depletion consideration in future pluripotent-derived neural populations. In this regard, we have found that CD133+/CD34- sorting has altered the *in vitro* fate profile of the hNSCs. Both the non-sorted and CD133+/CD34- populations similarly expressed neural stem/ progenitor markers Sox2 and Nestin under non-differentiating conditions. However, the sorted population, under differentiating conditions, appeared to be highly enriched for neurons. One possible explanation could relate to the developmental stage of the hNSCs at the time of cell sorting. Recent evidence has suggested that the neuralization method utilized in this study generates very early stage, pre-rosette hNSCs (Ebert et al., 2013). By sorting for the stemness marker CD133 at this early stage, we may have enriched the cell population for a more mitotically active early neural stem cell subtype that readily generates neuronal lineage cells (Tropepe et al., 2001; Pruszak et al., 2007; Elkabetz et al., 2008; Koch et al., 2009; Li et al., 2011b; Falk et al., 2012; Yan et al., 2013). Supporting evidence exists for the derivation of early lineage neural stem or neuroepithelial-like stem cells that are continuously expandable in culture, express over 90% CD133 on the cell surface, and generate predominantly neurons (Koch et al., 2009; Falk et al., 2012). These cells, termed long-term self-renewing neuroepithelial-like stem cells or lt-NES cells, exhibit hindbrain specification but are amenable to regional patterning *in vitro*, engraft after intracranial transplantation and fire action potentials *in vivo*, and differentiate

predominantly into GABAergic neurons (Steinbeck et al., 2012). Similarly, the early stage hNSCs generated via the protocols described herein appeared to be largely hindbrain specified, though a small glial population was present at least early in culture (up to passage 7). However, upon intrahippocampal transplantation, CD133+/CD34- XF Shef6 hNSCs generate predominantly glutamatergic neurons. Regardless, the CD133+/CD34- hNSCs described here represent a population of stable, self-renewing, and neurogenic early stage neural stem-like cells that may be of benefit for researchers studying human neural development.

A second interesting finding was the increased *in vitro* proliferation rate of the sorted XF Shef6 hNSCs. CD133+/CD34- hNSCs have previously been shown to have enhanced neurosphere forming capabilities (Uchida et al., 2000). Additionally, some proliferative neuroepithelial cell populations express CD133 (Pruszak et al., 2007; Falk et al., 2012), and CD133+ fractions of cancer biopsies and cancer cell lines have been shown to have increased proliferative rates (Singh et al., 2003; Kelly et al., 2010). Here, we have demonstrated that CD133 enrichment of XF Shef6 hNSCs has dramatically increased their expansion rate, effectively shortening their doubling time from ~6 days (non-sorted XF Shef6 hNSCs) to ~1 day (CD133+/CD34- XF Shef6 hNSCs). One possible explanation for the noted sub-optimal degree of cell growth and expansion in the non-sorted Shef6 hNSCs could be heterogeneity in the proportion of slower-cycling or quiescent neural stem/ progenitor-like cells to faster-cycling neural stem- or transit amplifying-like cells. Additionally, non-neural epithelial or multipotent stromal cell types could be present in the non-sorted population, as evidenced by the increased level of Desmin mRNA present in the non-sorted Shef6 hNSCs compared to the CD133+/CD34- sorted hNSCs. The possibility

exists then that these slower-cycling, possibly non-neural cells could be maintaining an overall slower rate of proliferation via secretion of factors such as BMPs, which have been shown to decrease NSPC proliferation and induce cell cycle exit (Liu and Niswander, 2005; Mathieu et al., 2008). By either enriching for faster-cycling cells or by eliminating slower-cycling cells (or both) via CD133+/CD34- cell sorting, we have potentially disengaged the brakes, at least partially, on this proliferation and cell cycle governing system *in vitro*. Moving forward, additional replication of the above Xeno-Free transition and CD133+/CD34- sorting studies with multiple different cell lines and cell types, including human induced pluripotent stem cells, will be critical to fully evaluate the utility of the methods presented here across a wide range of human pluripotent and neural cell types.

However, unabated *in vivo* cell proliferation can be problematic and often has undesired consequences such as tumor formation. CNS transplantation of highly proliferative hESCs and immature neural progenitors (Brederlau et al., 2006; Seminatore et al., 2010), as well as known neural tumor-forming cell lines (Fogh et al., 1977; Pollard et al., 2009), result in severe over-growth of the host tissue transplant region. In this study, we transplanted XF Shef6 hESCs into the hippocampus of immunodeficient NOD-*scid* mice and find similar results to previous studies - namely, the hESCs proliferate extensively, differentiate into cell types of all three embryonic germ layers (form teratomas), and engulf nearly the entire transplant region (hippocampus). Conversely, CD133+/CD34- XF Shef6 hNSCs do not exhibit transplant region over-growth or non-neural lineage differentiation. Rather, hippocampal engrafted sorted XF Shef6 hNSCs tend to distribute more sparsely throughout the molecular layer, dentate gyrus, and CA3 hippocampal regions and project fasciculated axon bundles. Interestingly, using similar cell preparation and transplantation

methods, hippocampal transplanted non-sorted XF Shef6 hNSCs failed to survive when examined at 12-weeks post-transplant. This failure could be the result of a combination of different factors, including, but not limited to, poor cell survival immediately post-transplant (Okada et al., 2005), insufficient proliferation of engrafted cells (as opposed to CD133+/CD34- XF Shef6 hNSCs that are more proliferative *in vitro*), and/or delayed cell death (Steinbeck et al., 2012). Future investigation of both the interactions between the transplanted non-sorted cell population and the host transplant niche, as well as the mechanisms responsible for the observed differences in gene expression and proliferation rate between the non-sorted and CD133+/CD34- XF Shef6 hNSCs, will be necessary to enhance our understanding of neural stem cell biology and cell transplantation, and critically, additional replication of these findings and long-term survival studies (i.e. 12 months or greater) in the injured or diseased niche must be performed before final conclusions can be drawn in regards to cell safety for future human use.

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CHAPTER 3

Transplantation of human neural stem cells restores cognition in an immunodeficient rodent model of traumatic brain injury

Abstract

Traumatic brain injury (TBI) in humans can result in permanent tissue damage and has been linked to cognitive impairment that lasts years beyond the initial insult. Clinically effective treatment strategies have yet to be developed. Transplantation of human neural stem cells (hNSCs) has the potential to restore cognition lost due to injury, however, the vast majority of rodent TBI/hNSC studies to date have evaluated cognition only at early time points, typically less than 1 month post-injury and cell transplantation. Additionally, human cell engraftment and long-term survival in rodent models of TBI has been difficult to achieve due to host immunorejection of the transplanted human cells, which confounds conclusions pertaining to transplant-mediated behavioral improvement. To overcome these shortfalls, we have developed a novel TBI xenotransplantation model that utilizes immunodeficient athymic nude (ATN) rats as the host recipient for the post-TBI transplantation of human embryonic stem cell (hESC) derived NSCs and have furthermore evaluated cognition in these animals at long-term (≥2 months) time points. We report that immunodeficient ATN rats demonstrate hippocampal-dependent spatial memory deficits at 2-3 months post-TBI, confirming that ATN rats recapitulate some of the cognitive deficits found in immunosufficient animal strains. hNSCs survived for at least 5 months post-transplantation and differentiated into cells from all three neural cell lineages. Furthermore, hNSC transplantation facilitated cognitive recovery after TBI, even in the

absence of gross histological modulation of lesion or total spared hippocampal tissue volume. Importantly, we have found an overall increase in host hippocampal neuron survival in hNSC transplanted animals and demonstrate that a correlation exists between hippocampal neuron survival and cognitive performance. Together, these findings support the use of immunodeficient rodents in models of TBI that involve the transplantation of human cells, and suggest that hNSC transplantation may be a viable, long-term therapy to restore cognition after TBI.

Introduction

Traumatic brain injury is a serious, debilitating condition that arises from an injury to the head due to blunt or penetrating trauma or from acceleration/deceleration forces. TBI affects upwards of 3.5 million people per year (Coronado et al., 2012), and there is an estimated 5.3 million people in the United States alone living with permanent, long-term TBI-related disabilities (Griesbach et al., 2015). However, these numbers likely underestimate the true incidence of TBI due to underreporting of sports- and military-related injuries, and persons who never seek healthcare (Roozenbeek et al., 2013). To date, treatment options for TBI focus on patient stabilization immediately after injury and subsequent long-term rehabilitative care. Considering that many pharmacologic interventions for TBI have been shown to be ineffective in clinical trials (Xiong et al., 2009; Maas et al., 2010; Lu et al., 2012), new treatment options must be explored. Stem cell transplantation has the potential to improve functional outcome after central nervous system (CNS) trauma and disease (Cummings et al., 2005; Xu et al., 2006; Ebert et al., 2008; Walker et al., 2009; Zhang and Chopp, 2009; Tetzlaff et al., 2011; Lindvall et al., 2012; Chen

et al., 2014; Rosser and Svendsen, 2014; Thomsen et al., 2014; Levy et al., 2015; Tsai et al., 2015; Wang et al., 2016). In the context of TBI, the transplantation of bone-marrow derived mesenchymal/stromal cells has been extensively studied (Li and Chopp, 2009; Aertker et al., 2015) and several clinical trials utilizing human mesenchymal stem cells have been approved and are upcoming (NCT02525432, NCT02416492), ongoing (NCT02028104, NCT01851083) or have been completed (NCT00254722, NCT01575470). However, transplanted human mesenchymal/stromal cells typically do not survive long-term in TBI rodent models (Gold et al., 2013). Poor cell survival, a rejection response, and/or failure to quantify cell survival can hinder the ability to accurately assess long-term cellular contribution to repair and recovery when cell replacement is a putative mechanism of action.

To enable the evaluation of continuous, long-term repair in animal models, the survival of transplanted cells must be improved. To achieve both increased and prolonged transplanted human cell survival in animal models of CNS injury and disease, the use of immunodeficient rodents (such as the NOD-*scid* mouse and athymic nude or ATN rat) has been shown to support enhanced transplanted human cell survival (Nasonkin et al., 2009; Anderson et al., 2011; Gowing et al., 2014; Mattis et al., 2014; Xu et al., 2015). Additionally, transplantation of phenotypically similar CNS-derived neural stem cells into the brain or spinal cord, as opposed to transplantation of foreign blood- or bone marrow-derived stem cells, may also improve transplanted cell survival. Therefore, in this study we have utilized immunodeficient ATN rats as our TBI model and transplanted previously characterized hESC-derived NSCs that have undergone selective cell sorting to enrich for the stemness marker CD133, a process that was shown to increase both *in vitro* expansion potential and

neuronal differentiation without increasing the risk of teratoma formation *in vivo* (Haus et al., 2014).

In addition to achieving improvements in transplanted human cell survival, there is also a need for future TBI and cell transplantation studies to be evaluated at long-term time points (≥2 months). Assessing only early cell survival and behavioral recovery poses two problems: 1) If hNSC engraftment and subsequent integration is integral to behavioral recovery, evaluation at later time points becomes critical as transplanted human cells will likely require at a minimum several months to fully integrate and differentiate into mature neural cell types, and 2) If long-term behavioral improvement is the desired clinical outcome, evaluation of recovery within days to weeks of injury or treatment is not informative for long-term behavioral gains. However, the majority of studies conducted to date assessing the effect of hNSC transplantation into rodent models of TBI have evaluated transplanted cell survival and behavioral recovery only at very early (typically 1-2 weeks) time points post-injury or cell transplantation (Gold et al., 2013).

Therefore, the objectives of this study were to first assess whether and what type of long-term (≥2 months) cognitive behavioral impairments exist post-TBI in an immunodeficient rodent model of TBI (utilizing the ATN rat). Second, we tested the hypothesis that intracranial transplantation of human embryonic stem cell-derived neural stem cells can promote long-term histopathological and cognitive behavioral improvement after TBI.

Materials and Methods

Animal Use and Controlled Cortical Impact (CCI) TBI

All animal housing conditions, surgical procedures, and postoperative care was approved by and conducted according to the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of California, Irvine. Adult male athymic nude (ATN) rats (NCI RNU -/- homozygous, 10-11 weeks old; Charles River Laboratories, San Diego, CA) were housed 2 per cage on a 12 hour light/dark cycle with free access to food and water. Unilateral cortical contusions were carried out under isoflurane anesthesia (3%) with rats positioned in a stereotaxic holder (Leica Microsystems Inc.). Injuries were produced using a pneumatic controlled cortical impact device (TBI 0310; Precision Systems and Instrumentation, Fairfax Station, VA) with a 5 mm flat metal impactor tip. The depth of the impact was set at 2.5 mm with a velocity of 4.5 m/sec and a dwell time of 500 msec. TBI coordinates were: A/P -4.5 mm, M/L -3.6 mm (left cortex, centered over hippocampus); prior to impact, a 6mm bone flap was removed using a manual trephine.

All animals received lactated ringers (50 ml/kg) subcutaneously immediately after surgery as well as Buprenorphine (0.5 mg/kg) immediately after surgery and for 2 days thereafter. Additionally, due to the immunodeficient nature of ATN rats, an antibiotic (Baytril, 2.5 mg/kg) was administered immediately after surgery and daily for 5 days thereafter.

Human Neural Stem Cell Preparation and Transplantation

Culture of Xeno-Free CD133+/CD34- Shef6 hNSCs was established at UC Irvine as previously described (Haus et al., 2014) and in accordance with all appropriate hSCRO and IBC protocols. hNSC passage number at the time of transplantation was either passage 7 (P7, “low passage”) or passage 27 (P27, “high passage”). All hNSC and vehicle preparations

took place during the day of transplantation/injection. All cells were maintained under normal culture conditions prior to harvesting and collection for transplantation. For hNSC injections, cells were dissociated to single cells using TrypLE and resuspended in X-Vivo 15 to yield $7.5x10^4$ cells/µl. Vehicle solution consisted of X-Vivo 15 cell culture media. During surgical procedures, all hNSC and vehicle preparations were kept at room temperature.

Animals were randomly allocated to different groups: sham (n = 10), vehicle injection (n = 12), and hNSC transplantation (n = 13 Shef6 P27, n = 11 Shef6 P7) and were processed blind to injection cohort. All hNSC and vehicle injections occurred 9 days post-TBI. Previously injured ATN rats were anesthetized with 3% isoflurane and positioned in a stereotaxic holder (Leica Microsystems Inc.). A Dremel rotary tool set at ~5,000 rpm was used to create injection burr holes. A 5 μl Hamilton syringe (Cat #87930) with a 1" 30G blunt needle was mounted into an UMP-3 (World Precision Instruments) injector connected to a SYS-Micro4 Controller. A total of four injections of either hNSCs or vehicle were made at the following coordinates: 1) Anterior Hippocampus (A/P -2.50, M/L +1.20, D/V -3.70), 2) Anterior Corpus Callosum (A/P -2.50, M/L +1.20, D/V -2.45), 3) Posterior Corpus Callosum (A/P -5.05, M/L +1.00, D/V -2.90), and 4) Posterior Hippocampus (A/P -5.05, M/L +4.00, D/V -3.90). Cells were first triturated 5 times with a 10 µl pipette then pulled up into the syringe over approximately 10 seconds. For each injection, the needle was initially lowered an additional 0.15mm to create a pocket in the brain. For each of the four injection sites, cells were injected at a dose of $6.25x10^4$ cells (at $7.5x10^4$ cells/µl) over 2 minutes (for a total dose of $2.5x10^5$ cells). Speeds varied relative to total volume, but time of injection remained constant. The needle was raised out of the brain 4 minutes after injection. The

syringe was tested to make sure it was not clogged by ejecting some of the solution into the air manually through the Micro4 controller. Bone wax was applied to seal the burr hole.

Animals received lactated ringers (50 ml/kg) subcutaneously immediately after surgery, Buprenorphine (0.5 mg/kg) immediately after surgery and for 2 days thereafter, and Baytril (2.5 mg/kg) immediately after surgery and daily for 5 days thereafter. No immunosuppressant drugs were administered.

Cognitive Behavioral Analysis

Novel Place and Object Recognition

Behavioral setup and analysis was based on previously published protocols (Acharya et al., 2009). All testing was done by experimenters blind to treatment group. Two open field plastic arenas, each measuring 45 x 70 x 70 cm were used for novel place recognition (NPR) and novel object recognition (NOR) testing. The arenas were placed next to each other on the floor of a brightly lit, dedicated behavioral testing room. A video camera was centered on the ceiling above the arenas, and live tracking of the animals was achieved using Noldus Ethovision XT (Noldus Information Technology). For familiarization testing, two identical objects were placed in opposing corners of the open field. For NPR testing, 24 hours later one of these objects was moved to an open corner ('novel place'), while the other block remained at its former spatial location ('familiar place'). For NOR testing, 24 hours later the object placed in the open corner ('novel place') was removed and replaced with a new, unfamiliar object ('novel object'). Small pieces of white Velcro placed on the undersides of the objects and on the arena floor were used to secure the objects in

place during testing. Arenas and objects were cleaned with a 0.1% acetic acid solution between trials to minimize odor cues.

To minimize animal stress, the experimenter handled all animals daily for 1 week before starting NPR testing. For the familiarization phase, rats were placed in the arenas and allowed to explore freely for 5 min. For the retention phases, rats were then returned to their home cages for 24 hours before being placed in the test arena again for the test phase, during which they were allowed to explore freely for 3 min. For all phases, the "head direction to zone" function in Ethovision XT was used to track exploration of the objects. A rat was considered to be exploring a block when its head was oriented toward it and its nose was within a 4-cm radius.

Discrimination index, calculated as the ratio of total time spent exploring the novel spatial location or novel object in relation to the familiar spatial location or object (tnovel-tfamiliar/tnovel+tfamiliar) was used as the main dependent measure. Previous research has shown that preference for the novel place can diminish after the first 1-2 minutes of testing, as the spatial locations become equally familiar to the animals (Mumby et al., 2002). During our NPR analysis we observed similar results; therefore, data from only the first 2 minutes of the 3 minute test phase was included due to the diminished preference for the novel place during the third minute of testing. Animals that did not explore either object during testing were excluded from analysis.

Morris Water Maze Acquisition and Reversal

The Morris water maze (MWM) consisted of a circular fiberglass pool filled with heated water made opaque by adding non-toxic white paint. A video camera suspended

above the pool recorded the animals and video output was recorded by an EthoVision tracking system (Noldus, Leesburg, VA). Visual cues were placed on the four walls surrounding the pool. The water maze protocol included 5 phases: cued learning, spatial acquisition training, probe test, spatial reversal training, and a second probe test. Twenty-four hours prior to spatial acquisition training, the animals were allowed to adapt to the maze for 60 seconds in a cued learning phase in which the hidden platform was flagged to enable easy location and visual cues were removed from the surrounding walls.

Spatial acquisition training: Twenty-four hours following cued learning, the animals were placed back into the same pool with the flag removed from the hidden platform and visual cues placed back onto the surrounding walls. The animals were then required to swim to find a hidden platform submerged just below the water surface. Training consisted of 4 trials per day for 5 consecutive days. For each trial, the animals were placed into the water facing the pool wall at one of four different starting positions (N, S, E, and W) chosen in semi-random order. During each trial, the rats were allowed to swim freely until they reached the hidden platform and remained for 10 seconds. Rats that failed to find the hidden platform in 60 seconds were guided to the platform location. Probe test: Twenty four hours after the final spatial acquisition training trial, the rats were returned to the pool and placed in a new location with the hidden platform absent for 60 seconds. The rats swim path and number of times crossing the former platform location was recorded.

Spatial reversal training: After the spatial acquisition learning task and initial probe test was completed, a reversal learning protocol was conducted. During reversal learning, the hidden platform was moved to the opposite quadrant. Reversal learning consisted of 5 additional days of training with 4 trials per day, similar to spatial acquisition training. Probe

test: Twenty four hours after the final reversal training trial, the rats were returned to the pool and placed in a new location with the hidden platform absent for 60 seconds. The rats swim path and number of times crossing the former platform location was recorded.

Elevated Plus Maze

The elevated plus maze (EPM) consisted of two open and two closed arms fabricated from black plastic (Med Associates Inc.). The apparatus was situated in a dark room, and experiments were recorded using infrared beam detection and an infrared video camera suspended above the plus maze. Live tracking of the animals was achieved using Noldus Ethovision XT (Noldus Information Technology). Each rat was placed in the center of the plus maze facing one of the open arms and allowed to explore for a total of 5 minutes. Total time spent in the open and closed arms was recorded as well as open and closed arm entries (counted when the center point of the rat crossed into either the open or closed arms). The apparatus was cleaned prior to the introduction of each animal. Rats were tested on the maze in randomized order.

Conditioned Taste Aversion

Behavioral setup and analysis was based on a previously published protocol (Lopez-Velazquez et al., 2007). Prior to Conditioned Taste Aversion (CTA) testing, the rats were deprived of water for 24 hours and given drinking access for 10 minutes twice a day for 6 days. On Day 7 (acquisition) the water was replaced with saccharin solution (0.1%). 20 minutes later the rats were injected with LiCl (7.5 ml/kg i.p., 0.2 M) to induce gastric malaise or taste aversion. On Days 8-10 water was given in the morning and afternoon

sessions to obtain baseline consumption. Beginning on the morning of Day 11, the water given was substituted with a freshly made 0.1% saccharin solution to test aversion (Day 11) and then extinction (Days 12-22).

Immunohistochemistry

At 20 weeks post-transplantation, tissue collection and immunohistochemistry was performed as previously described (Hooshmand et al., 2009) with minor modifications. Briefly, all animals were anesthetized with a lethal dose of Euthasol (100 mg/kg, i.p.) and transcardially perfused with 50 ml of PBS, followed by 300-400 ml of 4% paraformaldehyde. Brains were carefully dissected and post-fixed overnight in a solution of 4% paraformaldehyde and 20% sucrose in PBS at 4°C, flash frozen at −65°C in isopentane (2-methyl butane), and stored at −80°C.

For cryosectioning and immunohistochemistry, frozen brains were embedded in Optimal Cutting temperature (O.C.T.) compound (Sakura Finetek USA, Inc.). 30 μm thick coronal sections were cut using a cryostat and sections mounted using a CryoJane tape transfer system (Leica Biosystems, Inc., Buffalo Grove, IL). All tissue sections were antigen-retrieved and immunostaining procedures were conducted at room temperature. For 3,3′-diaminobenzidine (DAB) immunohistochemistry, sections were first washed in 0.1M Tris followed by a 15 minute incubation in 0.3% hydrogen peroxide/methanol. For fluorescent immunohistochemistry, wash and blocking steps were performed using PBS rather than Tris. After a brief 0.1% TritonX-100 wash, sections were blocked for 1 hour with bovine serum albumin (BSA) and normal serum from the species in which the secondary antibody was raised. Sections were then exposed overnight to the appropriate primary antibody. The

primary antibodies used were: monoclonal mouse anti-SC121 (DAB-1:8,000, Fluorescent-1:500) (AB-121-U-050, Clontech/Takara), polyclonal rabbit anti-hNestin (1:1,000) (ABD69, Millipore), polyclonal rabbit anti-Olig2 (1:500) (AB9610, Millipore), polyclonal rabbit anti-NeuN (1:1,000) (ABN78, Millipore), monoclonal mouse anti-APC (CC1) (1:200) (OP80, Calbiochem), polyclonal goat anti-Doublecortin (1:200) (SC-8066, Santa Cruz Biotechnology), and polyclonal rabbit anti-Glial Fibrillary Acidic Protein, GFAP (1:1,000) (Z0334, DAKO). The next day, sections were incubated with either fluorescent or biotin-conjugated, purified IgG secondary antibody (1:500, Jackson Immunoresearch) pre-adsorbed against the species in which the primary was raised, followed by avidin-biotinylated peroxidase complex (ABC) using a Vectastain Elite ABC kit (Vector Laboratories, USA) prepared according to the manufacturer's recommendations (for DAB staining). After several washes, the signal was visualized with diaminobenzidine (DAB) (Vector Laboratories, USA). Sections were mounted onto slides and allowed to dry overnight at 37°C. DAB stained sections were lightly counterstained with Cresyl Violet. Fluorescent stained sections were counterstained with Hoechst 33342 (1:500, Life Technologies). All slides were coverslipped using Depex or Fluoromount-G (SouthernBiotech) mounting medium. Imaging was performed using a Zeiss Imager.M2 Apotome System (Carl Zeiss) and a Zeiss Observer.Z1 Spinning Disk System (Carl Zeiss).

Quantification of Histology

Lesion, Spared Tissue, and Ventricle Volume Analysis

Histological sections comprised of the entire rostral to caudal extent containing cortex (approx. A/P +5.16 to A/P -9.36) were used to estimate total brain volume, lesion

volume, spared tissue (hippocampus) volume, and lateral ventricle volume. Unbiased stereological analysis was performed using a Zeiss Imager.M2 Apotome System (Carl Zeiss) and Microbrightfield Stereoinvestigator version 11.01.1 (MBF Bioscience) Cavalieri probe. A section interval of 1/12 was utilized, placing adjacent sections analyzed at 360 µm apart from each other. Cresyl violet was used to counterstain tissue sections for histological structure identification. Contours were drawn at 2.5x objective outlining the brain regions of interest and within them counting grids placed, including left/right hemisphere (grid size: 2,000 µm), left/right hippocampus (500 µm), left/right lateral ventricle (350 µm), lesion (1,000 µm), and other non-tissue space (350 µm). The described analysis provided an average coefficient of error (CE, m=1) ratio of <0.05 for all regions quantified.

Hippocampal Neuron Survival Analysis

Histological sections comprised of the anterior dorsal hippocampus (approx. A/P -1.72 to A/P -4.56) were used to estimate total neuronal survival in both the injured hemisphere dentate gyrus and combined Cornu Ammonis (CA) pyramidal cell layers (consisting of regions CA1, CA2, and CA3) as well as the uninjured hemisphere dentate gyrus. Unbiased stereological analysis was performed using a Zeiss Imager.M2 Apotome System (Carl Zeiss) and Microbrightfield Stereoinvestigator (MBF Bioscience) Optical Fractionator probe. A section interval of 1/12 was utilized, placing adjacent sections analyzed at 360 µm apart from each other. Cresyl violet was used to stain tissue sections for histological structure identification. Contours were drawn at 2.5x objective outlining the brain regions of interest and neuronal identification and counting was performed at 100x magnification. Only large Nissl-positive cells containing well defined perikarya and dense

ribosomes were counted to limit quantification to neurons. For all regions analyzed, the counting frame utilized was 25x25 μm with a grid size of 150x150 μm. The dissector height was set at 13 μm with an upper and lower guard zone of 1 μm. The described analysis provided an average coefficient of error (CE, m = 1) ratio for all animals (n = 24-30) of 0.074 (injured DG), 0.075 (injured CA), and 0.057 (uninjured DG).

Transplanted Human Cell Survival Analysis

Histological sections comprised of the entire rostral to caudal extent containing cortex (approx. A/P +5.16 to A/P -9.36) were used to estimate total human cell survival. Unbiased stereological analysis was performed using a Zeiss Imager.M2 Apotome System (Carl Zeiss) and Microbrightfield Stereoinvestigator (MBF Bioscience) Optical Fractionator probe. A section interval of 1/12 was utilized, placing adjacent sections analyzed at 360 μm apart from each other. An anti-SC121 (human cytoplasm) antibody was used to identify human cells and Cresyl violet was used to counterstain tissue sections for histological structure identification. Contours were drawn at 2.5x objective outlining areas of human cell engraftment, and human cell identification and counting was performed at 63x magnification. The counting frame utilized was 50x50 μm with a grid size of 200x200 μm. The dissector height was set at 12 μm with an upper and lower guard zone of 1 μm. The described analysis provided an average coefficient of error (CE, m = 1) ratio for all hNSC transplanted animals (n = 22) of 0.23.

Transplanted Human Cell Fate Analysis

Analysis was performed using a Zeiss Imager.M2 Apotome System (Carl Zeiss) and Bitplane: Imaris Scientific 3D/4D Image Processing and Analysis (Bitplane AG). A section interval of 1/24 was utilized, placing adjacent sections analyzed at 720 μm apart from each other. Co-localization was performed using either antibodies specific for neurons (NeuN), astrocytes (GFAP), oligodendrocytes (Olig2), and undifferentiated cells (Nestin) along with an anti-SC121 (human cytoplasm) antibody. Hoechst 33342 was used as a nuclear counterstain. To estimate the approximate number of cells of each fate for each animal, a minimum of 3 semi-random images containing at least 10 human cells were analyzed. Each fate group consisted of 4-6 animals.

Statistical Analysis

All behavioral, histological, and stereological analyses were performed by experimenters blinded to the experimental groups. Statistical analyses were performed using Prism (Version 6.0b Software). Behavioral data for tasks comparing sham and TBI animals were analyzed via either unpaired one-tailed (NPR, NOR, MWM Probe, EPM) or multiple (CTA) *t*-tests or via a repeated measures two-way ANOVA (MWM Acquisition and Reversal), unless otherwise stated in the results section. *T*-test results reported as t_{df} = t-score, *P* value. For behavioral tasks comparing sham, TBI + vehicle, and TBI + hNSCs, analysis was performed using either unpaired two-tailed *t*-tests (NPR), repeated measures two-way ANOVA (MWM Acquisition and Reversal), or Pearson Correlation (MWM Slope) analysis. All stereological quantifications were analyzed via unpaired two-tailed *t*-tests. Errors are the standard error (SEM) of averaged results. Significance was defined as at least $P < 0.05$ for all statistical analyses.

Results

Immunodeficient Rats Exhibit Long-Term Cognitive Impairment Following TBI

To improve the long-term survival of transplanted hNSCs, we utilized immunodeficient ATN rats as hNSC recipients. Because zero studies to date have assessed the behavioral response of immunodeficient ATN rats to TBI, multiple cognitive measures were evaluated at delayed (greater than 2 months) time points post-injury (Figure 3.1), including: hippocampal-dependent spatial memory (Novel Place Recognition, Morris Water Maze), non-spatial memory (Novel Object Recognition), emotional/anxiety-related behavior (Elevated Plus Maze), and classical conditioning (Conditioned Taste Aversion).

Spatial memory deficits are a hallmark of hippocampal injury, and in our study, the injury location was centered on the cortex directly overlying the dorsal hippocampus. To verify the presence of hippocampal-dependent spatial memory deficits, we measured cognition on two separate measures, Novel Place Recognition (NPR) and the Morris Water Maze. On both of these measures, ATN TBI animals displayed long-term cognitive impairment, performing significantly worse than sham animals at greater than 2 months post-injury. First, cognitive function after TBI was evaluated using the NPR task which measures the amount of time an animal spends exploring an object located in a novel location compared to an identical object located in a familiar position. Our results demonstrate that ATN TBI exhibited spatial memory impairment via a decrease in novel location exploration (TBI vs. sham: $P < 0.001$; Figure 3.1A). Additionally, hippocampal-dependent spatial memory deficits were detected in ATN TBI animals on a second task, the Morris Water Maze (MWM). MWM latency to platform assessment during both the

acquisition (Figure 3.1C) and reversal (Figure 3.1E) learning phases revealed deficits in ATN TBI animals on spatial target location memory as these animals had significantly increased latency to platform times relative to sham animals. Furthermore, probe trial analysis resulted in similar findings with ATN TBI animals spending less time in the target quadrant during both the acquisition (TBI vs. sham: $P < 0.05$; Figure 3.1D) and reversal (TBI vs. sham: $P < 0.01$; Figure 3.1F) learning phases, indicating that ATN TBI animals did not remember the location of the platform as well as shams.

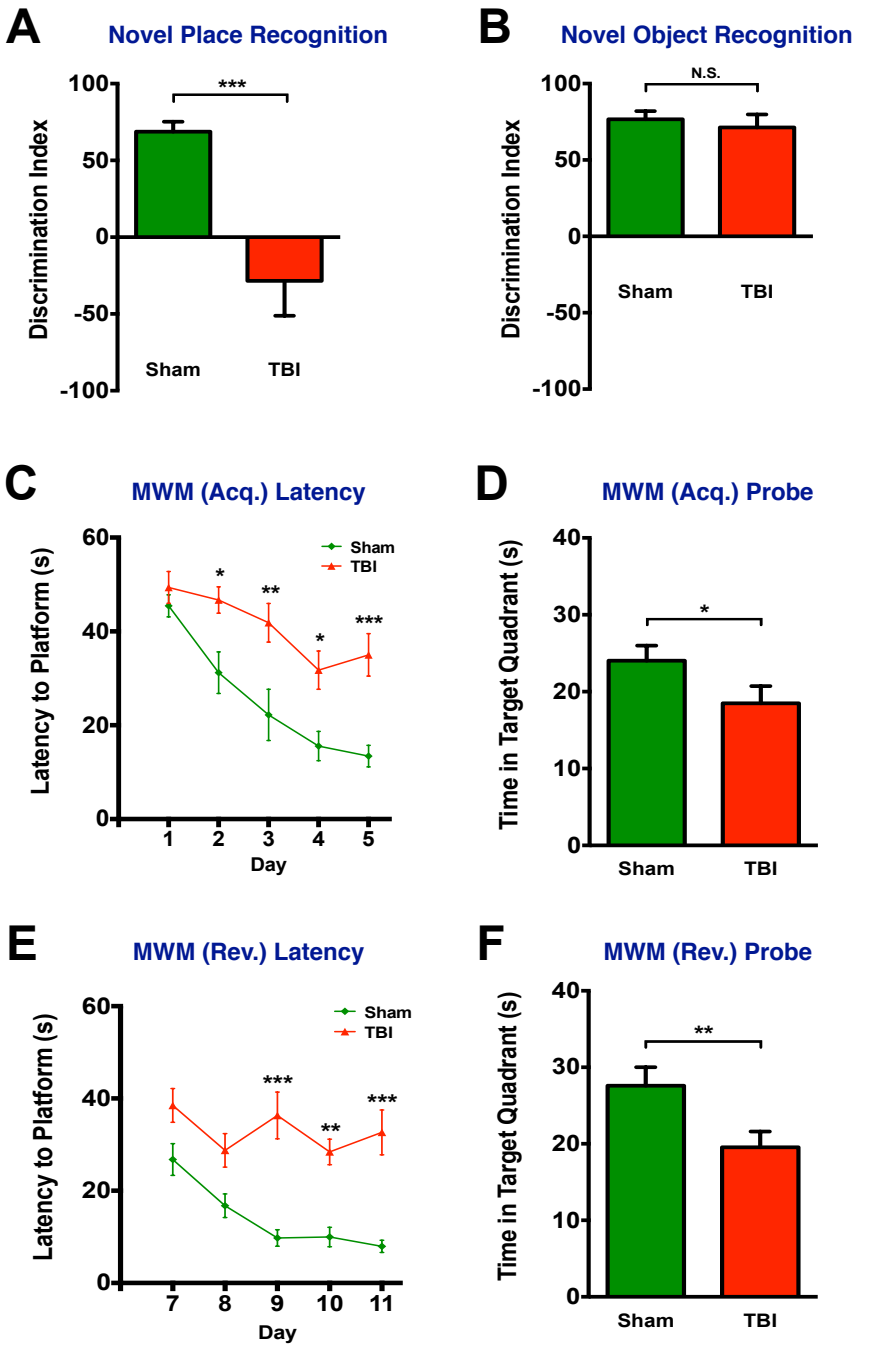

Figure 3.1. Controlled cortical impact TBI imparts long-term spatial memory impairments in immunodeficient ATN rats. Between 9-13 weeks post-TBI, animals were assessed for cognition via the spatial memory tasks Novel Place Recognition (NPR) and the Morris Water Maze (MWM), as well as the non-spatial memory task Novel Object Recognition (NOR). **(A)** TBI animals (n = 11) exhibited a significant 24-hour hippocampal-dependent NPR deficit during the first two minutes of testing (one-tailed *t* test $t19 = 3.93$, $P < 0.001$) when compared to sham animals (n = 10) that was not significantly different from zero (TBI, two-tailed *t* test $t10 = 1.25$, $P = 0.24$). **(B)** There was no difference between TBI (n = 12) and sham (n = 10) groups as

assessed via the 24-hour hippocampal-independent NOR task (one-tailed *t* test $t_{20} = 0.51$, $P = 0.31$). **(C)** MWM acquisition analysis revealed that on Days 2-5 TBI animals had significantly increased escape latencies finding the hidden platform relative to sham animals. Two-way repeated measures ANOVA was significant for trial day ($P < 0.0001$) and group ($P < 0.001$), but not for trial x group interaction ($P = 0.06$). **(D)** On Day 6, a Probe Trial revealed that TBI animals spent significantly less time in the target quadrant compared to sham animals (one-tailed *t* test $t_{20} = 1.83$, $P < 0.05$). **(E)** The platform location was then moved to a new location to test reversal learning. MWM reversal analysis revealed that on Days 9-11, TBI animals had significantly increased escape latencies finding the hidden platform relative to sham animals. Two-way repeated measures ANOVA was significant for trial day ($P < 0.0001$), group ($P < 0.0001$), and trial x group interaction ($P < 0.01$). **(F)** On Day 12, a Probe Trial revealed that TBI animals spent significantly less time in the target quadrant compared to sham animals (one-tailed *t* test $t_{20} = 2.55$, $P < 0.01$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean ± s.e.m.

TBI ATN rats were also assessed behaviorally on tasks that measure non-spatial memory, or on tasks that measure emotional/anxiety-related behavior or classical conditioning. Previous findings suggest that non-hippocampal or non-spatial memory impairments are present after TBI, however definitive long-term studies in a controlled cortical impact (CCI) model of TBI have not been conducted in either immunosufficient or immunodeficient animal models. Accordingly, we measured non-spatial memory via the Novel Object Recognition (NOR) task, emotional/anxiety-related related behavior via the Elevated Plus Maze (EPM), and classical conditioning via Conditioned Taste Aversion (CTA). Our results indicate that CCI TBI at the severity parameters utilized did not create any apparent long-lasting non-spatial, emotional/anxiety-related, or classical conditioning-related behavioral impairments. No statistically significant differences were found between ATN TBI and sham operated animals in novel object exploration times relative to a familiar object in the NOR task (TBI vs. sham: $P = 0.31$; Figure 3.1B). Additionally, no differences were found between ATN TBI and sham animals in the amount of time spent in the open

arms of the EPM (TBI vs. sham: P = 0.10; Figure 3.2A) or in the number of entries into the open arms (TBI vs. sham: P = 0.08; Figure 3.2B). Finally, no significant differences were demonstrated between ATN TBI and sham operated animals on the CTA task (Figure 3.2C), which was employed as an additional measure of classical conditioning to determine if TBI animals would exhibit impairment in associating a noxious event (LiCl-induced gastric malaise associated with saccharin consumption) with an everyday activity (water/ saccharin consumption).

Together, these results suggest that following a single, moderate-to-severe unilateral CCI TBI to the hippocampus and overlying cortex, immunodeficient ATN rats exhibit long-term hippocampal-dependent spatial memory deficits (NPR, MWM). In contrast, ATN performance on non-spatial or non-hippocampal-dependent memory tasks (NOR, EPM, CTA) remains indistinguishable from sham when tested at time points greater than 2 months post-TBI.

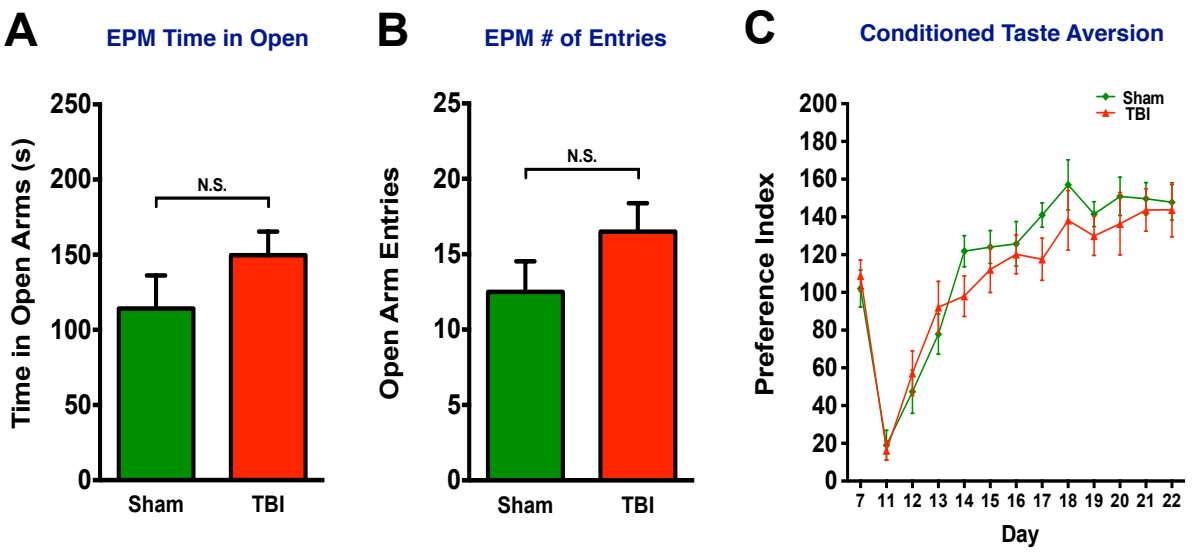

Figure 3.2. Controlled cortical impact TBI does not cause long-term emotional/anxiety-related memory impairment in immunodeficient ATN rats. Between 10-16 weeks post-TBI, animals were assessed via the Elevated Plus Maze (EPM) in a dark room and Conditioned Taste

Aversion (CTA). No detectable differences were found between TBI and sham animals on the EPM task as assessed via **(A)** total time spent in the open arms (one-tailed *t* test $t_{20} = 1.35$, $P = 0.10$) or **(B)** total number of open arm entries (one-tailed *t* test $t_{20} = 1.44$, $P = 0.08$). **(C)** Similarly, no differences between TBI and sham groups were found in the amount of 0.1% saccharin/water consumed after saccharin/LiCl administration on Day 11. Data are presented as mean ± s.e.m.

hNSC Transplantation Improves Long-Term Cognitive Recovery Following TBI

We next sought to determine whether hNSC transplantation could improve cognitive performance following TBI. To test this hypothesis, Xeno-Free CD133-enriched hNSCs were transplanted 9 days post-TBI into 4 sites (anterior medial corpus callosum, posterior medial corpus callosum, anterior dorsal hippocampus, posterior dorsal hippocampus) on the contralateral (uninjured) hemisphere of immunodeficient ATN rats. Following transplantation, animals were assessed behaviorally using the same hippocampal-dependent spatial memory tasks as assessed previously (NPR, MWM).

In the NPR task at 8-weeks, ATN TBI animals receiving vehicle injections performed significantly worse compared to sham animals (TBI + vehicle vs. sham: $P < 0.01$; Figure 3.3A). Transplantation of high passage (P27) CD133-enriched hNSCs rescued this performance deficit as animals receiving high passage hNSCs performed significantly better than ATN TBI animals that received vehicle injection only (TBI + Shef6 P27 hNSCs vs. TBI + vehicle: $P < 0.05$; Figure 3.3A). Furthermore, analysis of ATN TBI animals transplanted with high passage Shef6 hNSCs demonstrated a discrimination index that was statistically indistinguishable from sham-operated ATNs on the NPR task (TBI + Shef6 P27 hNSCs vs. sham: $P = 0.11$; Figure 3.3A). However, ATNs transplanted with low passage (P7) CD133-enriched hNSCs did not exhibit significantly improved cognition post-TBI when compared

to vehicle injected ATN TBI animals (TBI + Shef6 P7 hNSCs vs. TBI + vehicle: $P = 0.07$; Figure 3.3A), suggesting possible differences in behavioral rescue potential exist within the same cell line at different passage numbers.

Following TBI, ATN rats also demonstrated spatial memory impairment on the MWM task. However, hNSC transplanted ATNs did not demonstrate overtly improved cognition as measured by the amount of time (latency) needed to reach the submerged platform over five consecutive days of either acquisition learning (Figure 3.3B) or reversal learning (Figure 3.3D) as analyzed by a two-way repeated measures ANOVA. However, when the animals intra-group learning curves were assessed over each of the 5 day learning and reversal trials and plotted as group learning slopes (Rekha et al., 2009), a significant shift towards hNSC transplant-mediated cognitive improvement emerged. Whereas during the acquisition learning phase all groups exhibited negative (improved) learning slopes ($P < 0.001$; Figure 3.3C), in the reversal learning phase, ATN TBI animals receiving vehicle injection exhibited a group learning slope that was statistically equal to zero, indicative of a failure to learn the new platform location ($P = 0.36$; Figure 3.3E). In contrast, hNSC transplantation appeared to ameliorate this reversal relearning impairment as demonstrated by the negative learning slopes for both sham and low (P7) passage hNSC ($P < 0.001$; Figure 3.3E) as well as high (P27) passage hNSC ($P < 0.01$; Figure 3.3E) transplanted animals, further demonstrating that ATN TBI animals transplanted with hNSCs are better able to learn novel locations. No differences were observed in Probe trials for either the acquisition or reversal learning phases, regardless of group (data not shown).

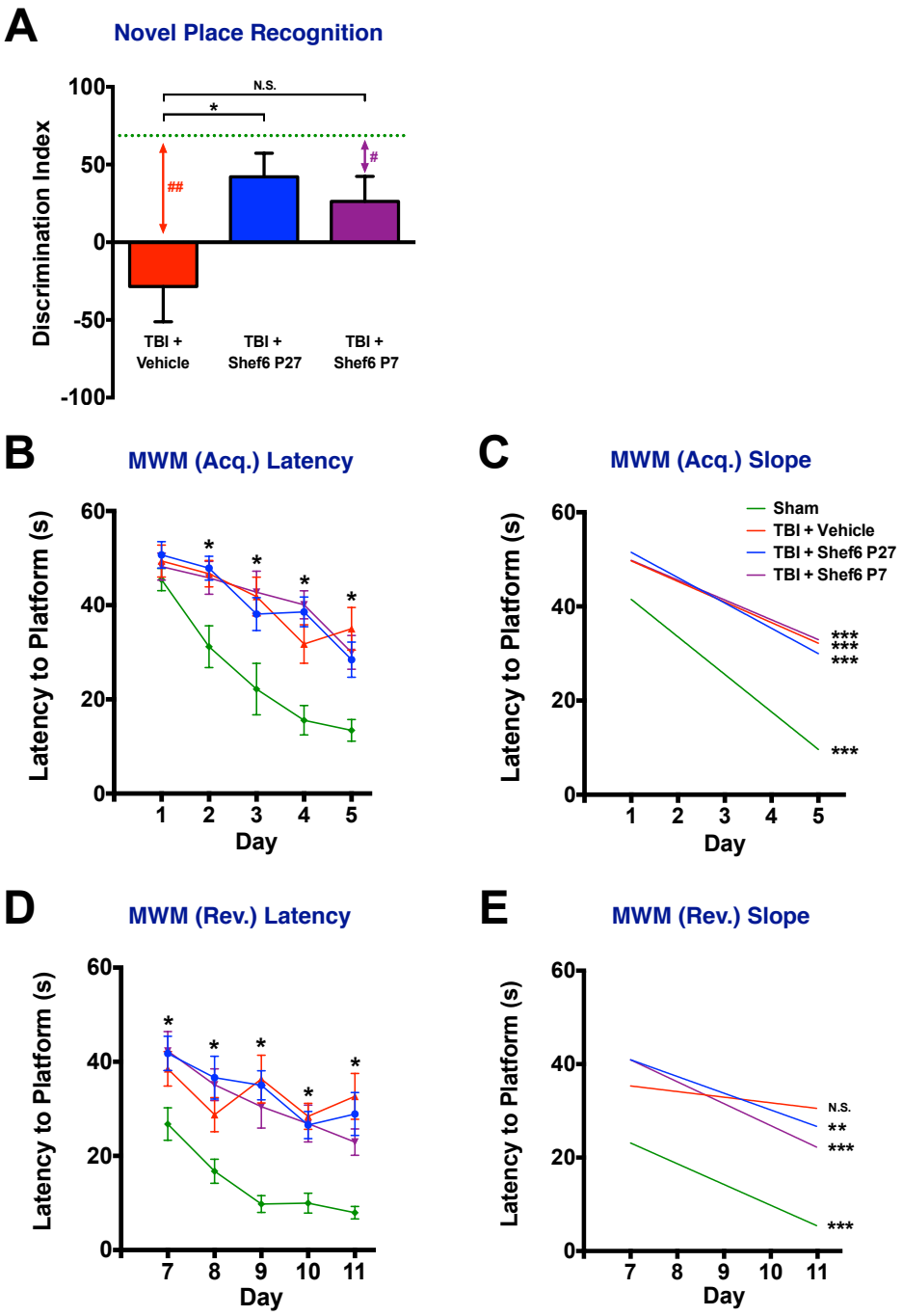

Figure 3.3. Transplantation of hNSCs improves cognitive behavioral outcomes after TBI. Between 8-12 weeks post-transplantation (9-13 weeks post-TBI), animals were assessed for improvements in cognition via both NPR and MWM. **(A)** On the NPR task, TBI animals given vehicle injections only (n = 11) performed significantly worse than sham animals (n = 10, two-tailed *t* test $t10 = 4.27$, $P < 0.01$). TBI animals that received high passage (P27) hNSCs (n = 11) exhibited a significant increase in their novel place exploration ratio during the first two minutes of testing as compared to TBI animals that received vehicle injections only (two-tailed *t* test $t20 = 2.58$, $P < 0.05$). TBI + Shef6 P27 hNSC animals were indistinguishable from sham animals

(two-tailed *t* test $t10 = 1.76$, $P = 0.11$) and significantly different from zero (two-tailed *t* test $t10 = 2.78$, $P < 0.05$). TBI animals given low passage (P7) hNSCs (n = 10) did not exhibit increased exploration compared to vehicle injected TBI animals (two-tailed *t* test $t19 = 1.93$, $P = 0.07$) and performed significantly worse than sham animals (n = 10, two-tailed *t* test $t9 = 2.63$, $P < 0.05$). **(B)** Analysis of MWM acquisition escape latency revealed that on Days 2-5, TBI animals given vehicle injection or hNSCs had significantly increased escape latencies finding the hidden platform relative to sham animals. Two-way repeated measures ANOVA was significant for trial day ($P < 0.0001$), group ($P < 0.0001$), and trial x group interaction ($P < 0.05$). No significant differences were detected between TBI animals given vehicle injection only and TBI animals given hNSCs (P27 or P7). **(C)** Analysis of the slope of acquisition via linear regression revealed significantly non-zero slopes for all groups during MWM acquisition ($P < 0.001$). **(D)** MWM reversal escape latency analysis revealed that on Days 7-11, TBI animals given vehicle injection or hNSCs had significantly increased escape latencies finding the hidden platform relative to sham animals. Two-way repeated measures ANOVA was significant for trial day ($P < 0.0001$) and group ($P < 0.0001$), but not for trial x group interaction ($P = 0.11$). No significant differences were detected between TBI animals given vehicle injection only and TBI animals given hNSCs (P27 or P7). **(E)** Analysis of the slope of acquisition via linear regression revealed significantly non-zero slopes for sham and TBI animals given Shef6 P7 ($P < 0.001$) or Shef6 P27 hNSCs ($P < 0.01$) during MWM reversal, however TBI animals given vehicle injection only were statistically indistinguishable from zero ($P = 0.36$). All data is from analysis conducted 20 weeks post-hNSC transplantation/vehicle injection. # represents comparisons made to sham animals. * represents comparisons made between groups (A, B, D) or compared to zero slope (C, E). #/* $P < 0.05$, ##/** $P < 0.01$, ###/*** $P < 0.001$. Data are presented as mean ± s.e.m.

hNSC Transplantation Improves Host Hippocampal Neuron Survival Without Modification of Lesion or Total Spared Tissue Volumes

Because hNSC transplantation in our study improved hippocampal-dependent spatial memory, we sought to determine whether a histological correlate to behavioral recovery existed. First, to quantitatively assess the long-term impact of both TBI and the subsequent transplantation of hNSCs on lesion size, total spared tissue volume, and ventricle volume, detailed stereological analysis was performed using the Cavalieri method (Figure 3.4). Stereological analysis confirmed the presence of a unilateral lesion cavity occupying primarily somatosensory, motor, and visual cortex regions as well as the

underlying corpus callosum and hippocampus (Figure 3.4A-B) on the side of impact. No statistical differences in cortical or hippocampal volumes on the uninjured hemisphere were found for any group when compared to sham animals, confirming the unilateral nature of the injury (data not shown). Following hNSC transplantation, lesion volumes in both the low and high passage hNSC groups remained unchanged compared to ATN TBI animals given vehicle injection (Figure 3.4C), suggesting that hNSC transplantation in our study did not decrease lesion size.

Spared tissue and ventricle volume was also assessed. In the hemisphere ipsilateral to injury, hippocampal volumes decreased significantly post-TBI (TBI + vehicle vs. sham: $P < 0.001$; Figure 3.4D), with no volume differences detected between hNSC transplanted and vehicle injected animals. Along with hippocampal tissue loss, significant ventricle enlargement post-TBI was also detected (TBI + vehicle vs. sham: $P < 0.01$; Figure 3.4E), in some cases increasing in volume up to three-fold, with hNSC transplantation facilitating a downwards, but not statistically significant, trend in ipsilateral ventricular volume. Taken together, these results demonstrate that TBI at the parameters utilized leads to the formation of a persistent lesion cavity as well as decreased hippocampal tissue volume and ventricular enlargement in the hemisphere ipsilateral to injury. hNSC transplantation did not alter any of these volumetric outcome measures, yet still managed to effect behavioral recovery, suggesting the presence of an alternative mechanism(s).

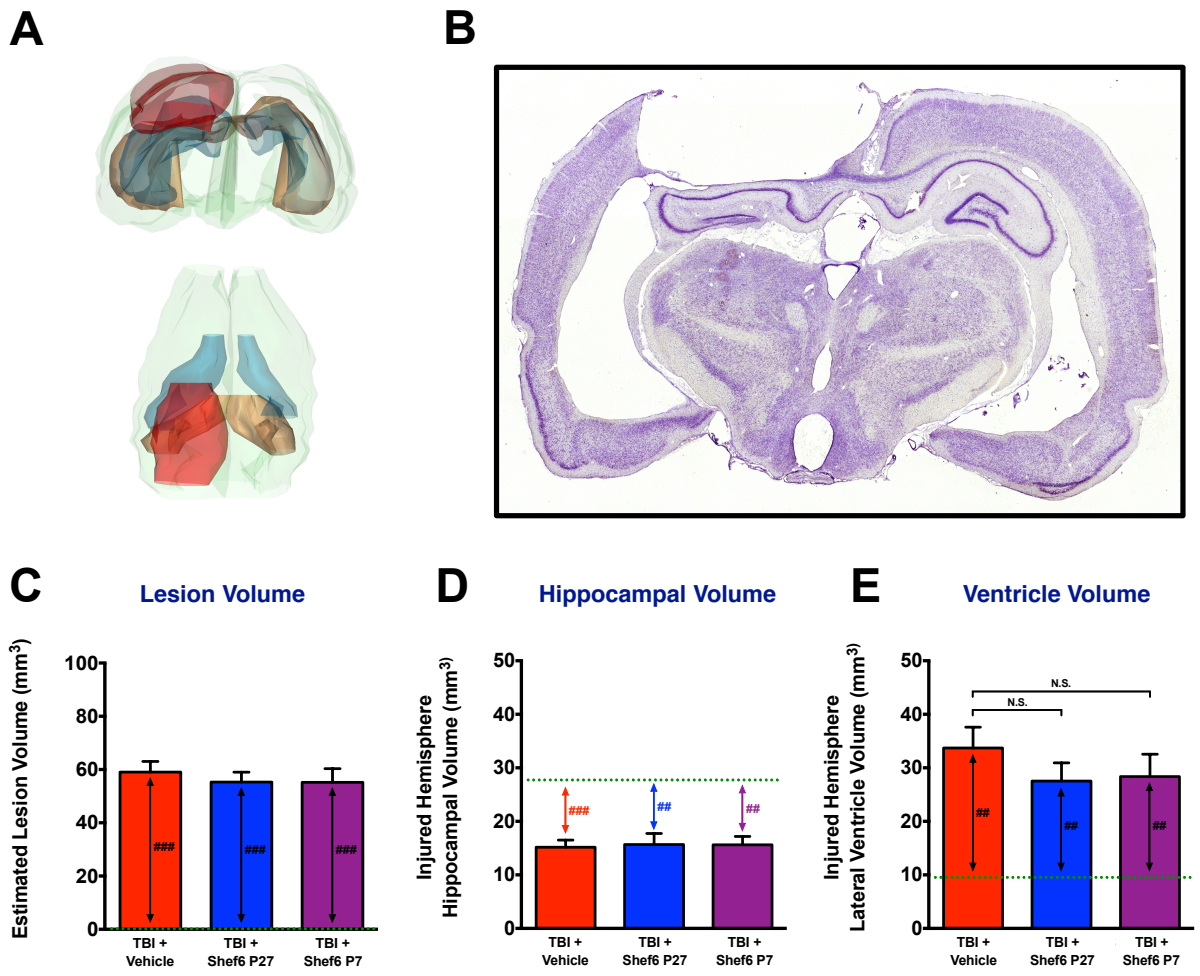

Figure 3.4. Controlled cortical impact TBI causes long-term tissue loss and ventricular swelling that is not resolved via hNSC transplantation. **(A)** 3-D volumetric reconstruction of lesion (red), hippocampus (orange), and ventricular space (blue) at 20 weeks post-transplantation. **(B)** Representative histological section depicting extent of long-term damage resulting from CCI-TBI. Volumetric analysis of **(C)** lesion volume demonstrates significant lesion formation in all TBI groups when compared to sham animals (two-tailed *t* test, $P < 0.0001$) with no change in lesion size as a result of hNSC transplantation. As a result of TBI, there is **(D)** a significant reduction in ipsilateral hippocampal tissue volume in vehicle injected animals when compared to sham (two-tailed *t* test $t5 = 7.74$, $P < 0.001$) as well as **(E)** a significant increase in ipsilateral lateral ventricle volume in vehicle animals when compared to sham (two-tailed *t* test $t5 = 6.18$, $P < 0.01$). hNSC transplantation did not alter either hippocampal tissue loss or lateral ventricle swelling. All data is from analysis conducted 20 weeks post-hNSC transplantation/vehicle injection. # represents comparisons made to sham animals. * represents comparisons made between groups. #/* $P < 0.05$, ##/** $P < 0.01$, ###/*** $P < 0.001$. Data are presented as mean ± s.e.m.

Volumetric quantification of affected brain regions after injury provides an important global view of histological outcome. However, subtle changes at the cellular level, such as increased host neuron survival, may be missed with this method. We therefore tested if hNSC transplantation affects long-term host hippocampal neuronal survival after TBI via stereological assessment of hippocampal neuronal survival in both the dentate gyrus (DG) and combined pyramidal cell layers (CA1, CA2, CA3; referred to as CA). In this study, ATN TBI animals that received vehicle injection had significantly fewer hippocampal neurons when compared to sham animals in both the DG (TBI + vehicle vs. sham: $P < 0.001$; Figure 3.5B) and CA (TBI + vehicle vs. sham: $P < 0.0001$; Figure 3.5C) regions ipsilateral to injury. Transplantation of high passage (P27) Shef6 hNSCs rescued host neuronal loss in both the DG (TBI + Shef6 P27 hNSCs vs. TBI + vehicle: $P < 0.05$; Figure 3.5B) and CA (TBI + Shef6 P27 hNSCs vs. TBI + vehicle: $P < 0.05$; Figure 3.5C) regions. No differences were found in host neuron survival on the uninjured (non-impacted side) DG for any TBI group compared to sham animals (data not shown). ATN TBI animals that received low passage (P7) Shef6 hNSCs demonstrated a non-significant trend towards increased host neuron survival in these same regions. In parallel, correlation analysis revealed positive relationships between both host DG neuron survival (Pearson $r = 0.70$, $P = 0.0003$; Figure 3.5D) and host CA neuron survival (Pearson $r = 0.64$, $P = 0.001$; Figure 3.5E) with performance on the NPR task, suggesting a strong relationship between hippocampal neuron survival and cognitive performance.

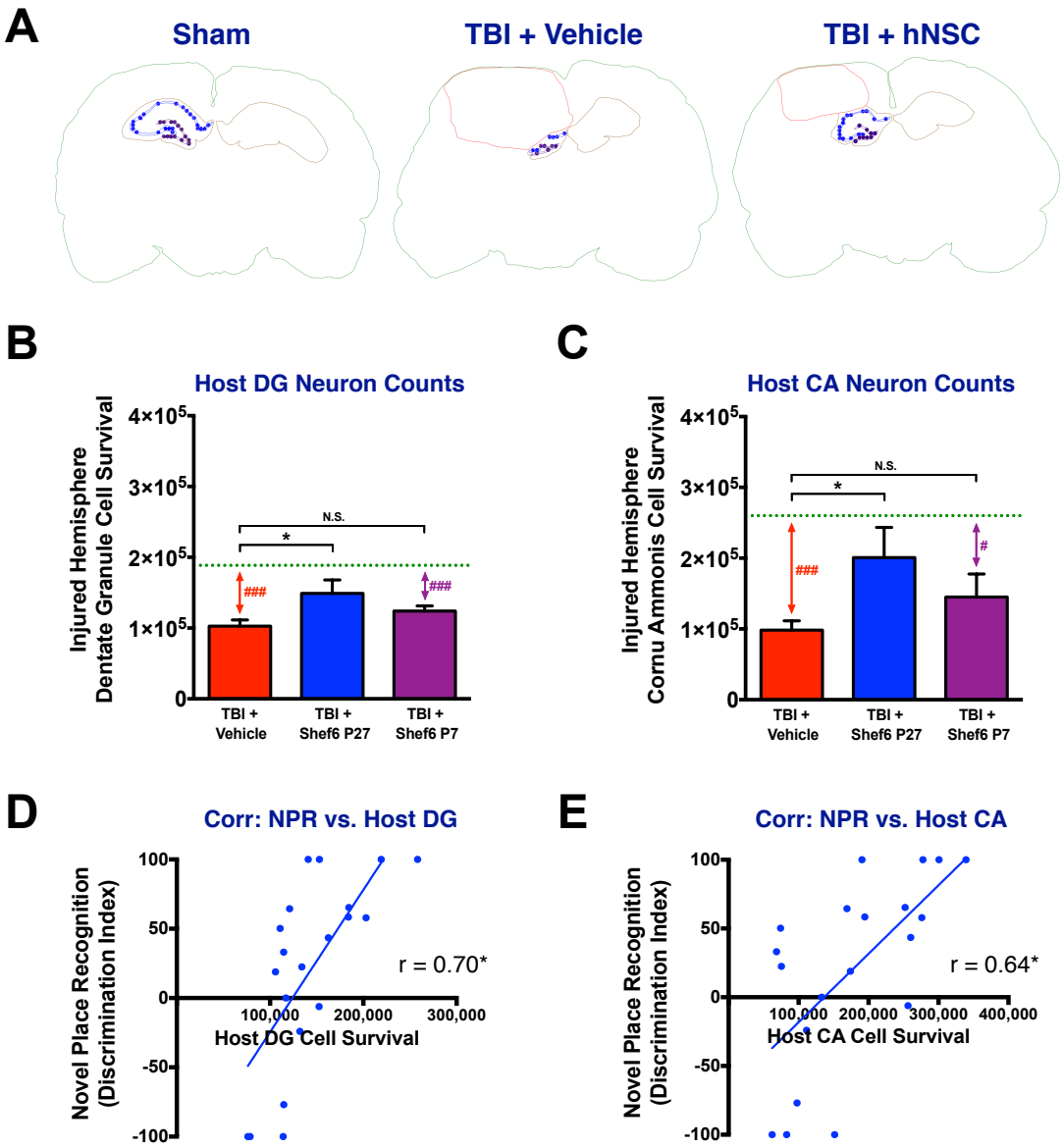

Figure 3.5. hNSC transplantation rescues host hippocampal neuron loss after TBI. (A) Representative image reconstructions of host neuron quantification in hippocampal dentate gyrus (DG) (purple circles) and cornu ammonis (CA) (blue circles) regions. Lesion is outlined in red. **(B-C)** Quantification of host hippocampal neuron survival in the injured DG and CA regions. When compared to sham animals, significant neuronal loss is present in vehicle injected TBI animals in both the **(B)** injured DG (two-tailed *t* test $t_5 = 9.76$, $P < 0.001$) and **(C)** injured CA (two-tailed *t* test $t_5 = 12.29$, $P < 0.0001$). When compared to vehicle injected TBI animals, transplantation of high passage (P27) Shef6 hNSCs prevents neuronal loss in both the **(B)** injured DG (two-tailed *t* test $t_9 = 2.33$, $P < 0.05$) and **(C)** injured CA (two-tailed *t* test $t_9 = 2.49$, $P < 0.05$). Transplantation of low passage (P7) Shef6 hNSCs had no effect on neuronal survival after TBI in either region. **(D-E)** Linear regression analysis revealed significant positive correlations between **(D)** host DG cell survival and NPR score (Pearson $r = 0.70$, $P = 0.0003$, one-tailed *t* test) and **(E)** host CA cell survival and NPR score (Pearson $r = 0.64$, $P = 0.001$, one-tailed *t* test).

All data is from analysis conducted 20 weeks post-hNSC transplantation/vehicle injection. # represents comparisons made to sham animals. * represents comparisons made between groups. #/* $P < 0.05$, ##/** $P < 0.01$, ###/*** $P < 0.001$. Data are presented as mean ± s.e.m.

Transplanted hNSCs Survive Long-Term and Differentiate Into Cells of All Three Neural Lineages

A major issue that exists in studies transplanting human cell populations into rodent models of TBI is low overall graft survival, likely due to human-to-rodent xenograft histocompatibility issues and graft rejection. Immunodeficient ATN rats, which are both T- and B-cell deficient, were therefore used in our study to limit the graft rejection response. At 20-weeks post-transplantation, surviving hNSCs (SC121+) exhibited engraftment throughout the host brain, extending into ventral hippocampal and ventral/medial cortical regions as well as occasionally crossing into the injured (non-transplanted) hemisphere (Figure 3.6A, D-E). Of the initial 250,000 hNSCs transplanted 9-days post-TBI, 21,846 ± 10,408 (or 8.7% ± 4.2) high passage P27 CD133-enriched hNSCs and 62,201 ± 17,397 (or 24.9% ± 7.0) low passage P7 CD133-enriched hNSCs survived long-term (Figure 3.6B). However, correlations of transplanted hNSC survival with cognitive improvements were not statistically significant (Figure 3.6C). Overall, while the total transplanted hNSC survival estimates in this study may seem low in comparison to that typically observed when using immunodeficient animals for human cell transplantation studies (Anderson et al., 2011), the extent of transplanted cell survival in this study demonstrates a dramatic improvement over previous reports of transplanted human neural cell survival in rodent models of TBI (Gold et al., 2013).

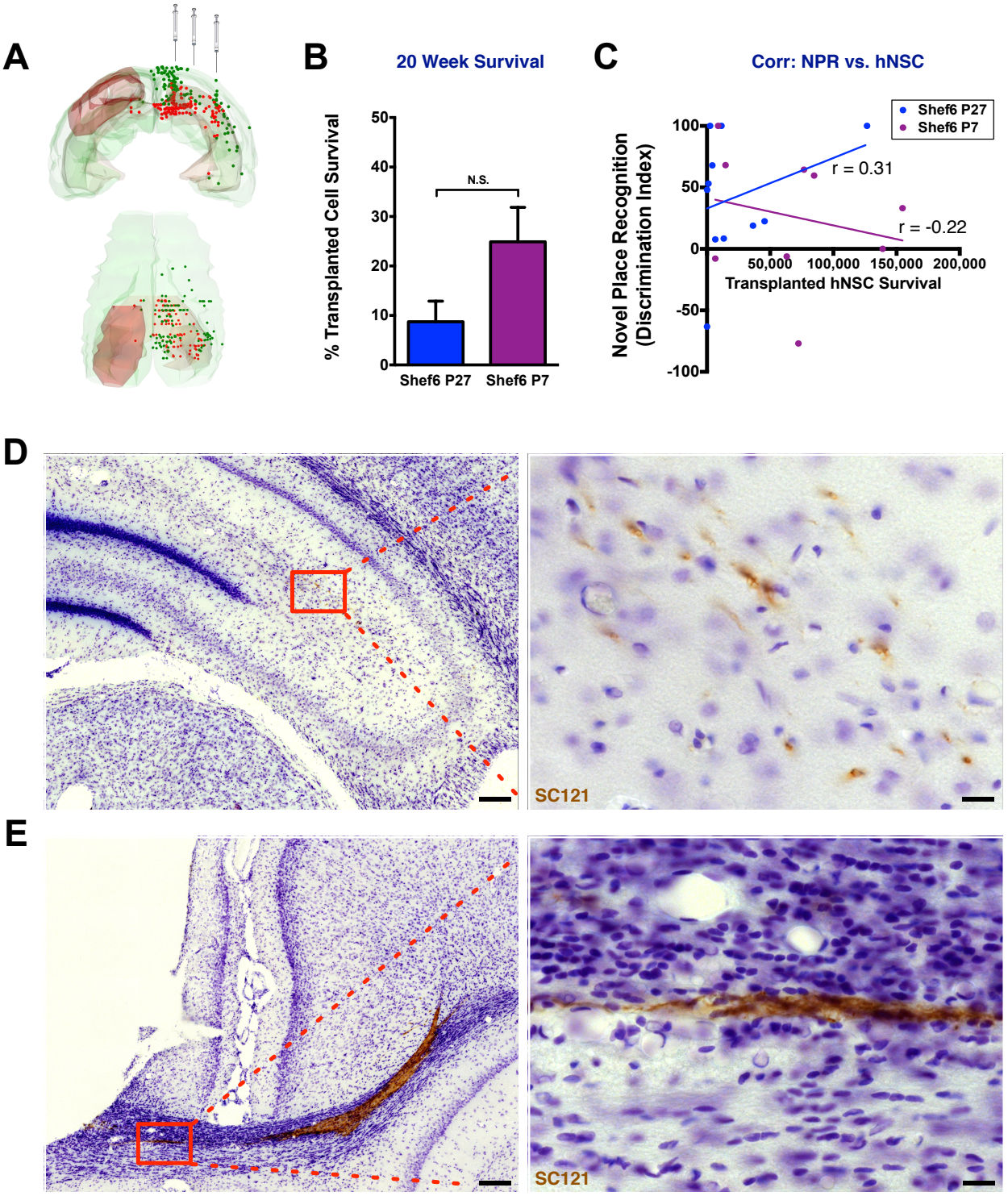

Figure 3.6. Transplanted hNSCs engraft and survive long-term in an immunodeficient rodent model of TBI. (A) 3-D reconstruction of the location of surviving hNSCs at 20 weeks post-transplantation that engrafted in either cortex (green circles) or hippocampus (red circles). Lesion is outlined in red. **(B)** Stereological quantification of transplanted high passage P27 Shef6 (n = 12, 8.7% ± 4.2) and low passage P7 Shef6 (n = 10, 24.9% ± 7.0) hNSC survival. No statistical difference in transplanted hNSC survival was detected between cell populations (two-

tailed *t* test t_{20} = 2.07, *P* = 0.052). **(C)** Linear regression analysis revealed no significant correlations between transplanted hNSC survival and NPR score (Shef6 P27, Pearson *r* = 0.31, *P* = 0.18; Shef6 P7, Pearson *r* = -0.22, *P* = 0.28, one-tailed *t* test). **(D-E)** Immunohistochemistry of transplanted hNSCs (SC121+) demonstrates survival and engraftment in both the **(D)** hippocampus and **(E)** corpus callosum. Red boxes delineate regions shown at higher magnification. Scale bars for low magnification images = 150 μm and for high magnification images = 15 μm. All data is from analysis conducted 20 weeks post-hNSC transplantation or vehicle injection. Data are presented as mean ± s.e.m.

Previously, we have found that when transplanted into a naïve immunodeficient host, the cell population used in this study differentiates predominantly into neurons (Haus et al., 2014). Similarly, cell fate analysis in this study demonstrated the predominant terminal fate of transplanted hNSCs was neuronal, with 18.6 ± 4.1% of high passage (P27) Shef6 hNSCs and 37.6 ± 2.0% of low passage (P7) Shef6 hNSCs differentiating into NeuN+ neurons (Shef6 P27 vs. Shef6 P7: *P* < 0.01; Figure 3.7A-B). However, a smaller percentage of both astrocytic (GFAP) (Shef6 P27, 13.2 ± 3.4%; Shef6 P7, 15.8 ± 3.2%; Figure 3.7C-D) and oligodendroglial (Olig2) (Shef6 P27, 11.3 ± 1.7%; Shef6 P7, 13.1 ± 2.5%; Figure 3.7E-F) differentiation was found to be present, suggesting that the TBI microenvironment may influence the *in vivo* differentiation potential of transplanted embryonic-derived hNSCs. Additionally, a significant portion of transplanted hNSCs remained in an undifferentiated (Nestin+) state, even at 20-weeks post-transplantation (Shef6 P27, 9.7 ± 6.3%; Shef6 P7, 23.9 ± 5.3%; Figure 3.7G-H).

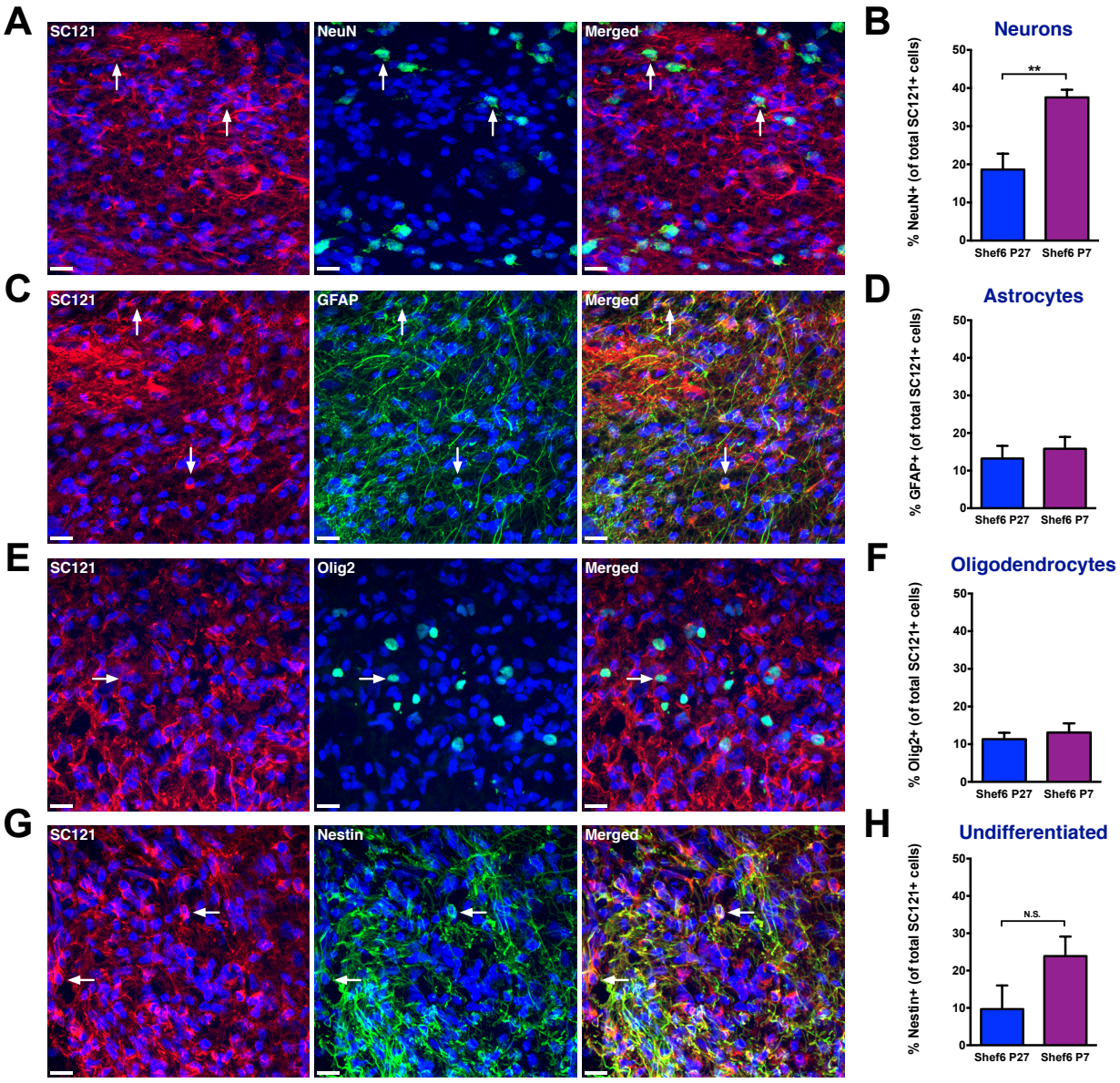

Figure 3.7. Transplanted hNSCs differentiate into all three neural lineage cell types. At 20 weeks post-transplantation, hNSCs differentiate into either **(A-B)** mature NeuN+ neurons (Shef6 P27, n = 5, 18.6% ± 4.1; Shef6 P7, n = 6, 37.6% ± 2.0; Shef6 P27 vs. Shef6 P7, two-tailed *t* test $t9 = 4.36$, $P < 0.01$), **(C-D)** GFAP+ astrocytes (Shef6 P27, n = 4, 13.2% ± 3.4; Shef6 P7, n = 6, 15.8% ± 3.2), and **(E-F)** Olig2+ oligodendrocytes (Shef6 P27, n = 5, 11.3% ± 1.7; Shef6 P7, n = 6, 13.1% ± 2.5), or remain **(G-H)** undifferentiated and express Nestin (Shef6 P27, n = 5, 9.7% ± 6.3; Shef6 P7, n = 6, 23.9% ± 5.3). White arrows indicate co-localized cells. Scale bars = 15 µm. All data is from analysis conducted 20 weeks post-hNSC transplantation/vehicle injection. ** $P < 0.01$. Data are presented as mean ± s.e.m.

Discussion

Validating long-term cognitive deficits in an immunodeficient rodent model of TBI.

TBI research in animal models to date has primarily focused on short term (hours to days) histological and behavioral outcomes (Marklund and Hillered, 2011; Xiong et al., 2013). While such studies may be informative for assessing the acute response to various forms of intervention, long-term (≥2 months) studies are necessary to fully examine long-lasting therapeutic efficacy and to ensure that any behavioral gains are due to treatment and not spontaneous recovery (Gold et al., 2013). Furthermore, the need for long-term studies is particularly important when the therapy being evaluated involves the transplantation of human neural stem cells that may require several weeks-to-months to integrate and mature *in vivo*.

Therefore, we first characterized a battery of potential long-term cognitive behavioral impairments following TBI in immunodeficient ATN rats. This is the first report of these measures in ATN rats using a controlled cortical impact (CCI) model of TBI. In this study, immunodeficient ATN rats demonstrated hippocampal-dependent spatial memory deficits at 2-3 months post-injury (NPR, MWM), thus confirming that cognitive deficits that are typically present after TBI in common immunosufficient animal strains are also present after TBI in immunodeficient ATN rats and persist for several months after injury. However, the same brain-injured animals did not demonstrate long-term cognitive impairment on either a non-spatial memory task (NOR) or on measures that test anxiety or emotional memory (EPM, CTA). Interestingly, previous studies have reported cognitive impairment after TBI on non-spatial memory or anxiety measures (Gold et al., 2013). However, the majority of these studies did not evaluate cognitive behavior at long-term (≥2 months) time

points after injury. Of the few studies that did evaluate behavior at time points greater than 2 months post-injury, one group reported EPM and Forced Swim impairments that were shown to persist until 2 months post-injury (Shultz et al., 2012; Shultz et al., 2013), and in a second set of studies, Forced Swim and Passive Avoidance impairments were present up to 2-3 months post-TBI (Milman et al., 2005; Milman et al., 2008). In each of these studies, however, the injuries were generated by diffuse methods (Lateral Fluid Percussion Injury and Weight-Drop, respectively). Conversely, our injury paradigm (CCI) generates a more focal injury in which brain regions critical for non-spatial or anxiety/emotional-related memory may not have been damaged to the same extent found in studies utilizing other injury methods, thereby generating shorter-lasting impairment. However, the alternative possibility that such memory deficits never existed from the outset in our study must also be considered as we only performed cognitive assessments at later time points.

Evaluating the effects of hNSC transplantation on cognitive recovery following TBI.

To evaluate the potential for hNSC transplant-mediated cognitive improvement post-TBI, hippocampal-dependent spatial memory was measured. Previous studies have shown efficacy for the transplantation of human neural cell types in ameliorating cognitive dysfunction after TBI, including the transplantation of fetal human neural stem/progenitor cells (Hagan et al., 2003; Al Nimer et al., 2004; Wennersten et al., 2004; Gao et al., 2006; Wennersten et al., 2006; Skardelly et al., 2011; Park et al., 2012; Poltavtseva et al., 2012; Wang et al., 2012) and human NT2N neurons (Muir et al., 1999; Philips et al., 2001; Watson et al., 2003; Longhi et al., 2004; Zhang et al., 2005). Critically however, only one of these studies evaluated cognitive function long-term (2 or more months) after injury/

transplantation and reported improvement on a cognitive measure (Skardelly et al., 2011), further demonstrating the need for more long-term cognitive behavioral studies to be conducted in the future. Additionally, the findings from many TBI and stem cell transplantation studies are based on methods that may be too heterogeneous to facilitate direct comparisons between studies and in some cases, publication bias may be present as larger studies with higher Quality Index (QI) scores often have smaller effect sizes than small studies with low QI scores (Chang et al., 2015).

While recent studies have reported the successful engraftment and survival of human pluripotent-derived stem cells in rodent models of TBI, including the demonstration of human induced pluripotent stem cell (hiPSC) derived NSC engraftment and cell tracking (Tang et al., 2013) as well as the first demonstration of successful hESC-derived oligodendrocyte progenitor cell (OPC) engraftment in an ATN TBI model (Xu et al., 2015), to our knowledge, zero studies to date have been conducted assessing the effects of hESC-NSC transplantation in modulating cognitive function after TBI. Our study therefore is the first demonstration of hESC-NSC transplant-mediated recovery of cognitive function post-TBI. ATN TBI animals transplanted with hNSCs demonstrated significant improvement on both the NPR and MWM tasks. However, the MWM improvement was observed only in the reversal phase and also only when learning slopes were analyzed, not latency to platform. The reversal phase of MWM is considered to be more challenging than acquisition as it requires animals to both extinguish a previously learned location and then learn a new one (Vorhees and Williams, 2006). Multiple examples exist where experimental animals show little to no differences during acquisition but exhibit strong deficits during reversal (Vorhees and Williams, 2014), particularly in the case of hippocampal damage. In our MWM

reversal assessment, sham animals from the outset quickly learned the new platform location. However, all ATN TBI animals took considerably longer, suggesting a slower learning process. By looking then at learning slopes, we can observe that ATN TBI animals given vehicle only never demonstrate any improvement (zero slope) during reversal testing. Conversely, ATN TBI animals transplanted with hNSCs do demonstrate improvement (negative slope), and their rate of learning (slope) is nearly identical to that of sham animals. Together with the improvement observed on the NPR task, these data suggest an overall net positive effect on cognitive improvement post-TBI in animals transplanted with hNSCs.

Evaluating the effects of hNSC transplantation on histological outcome measures following TBI.

In this study, unbiased stereological methods were used to quantify the effect hNSC transplantation has on modulating histological measures such as lesion volume, spared hippocampal volume, ventricle volume, and host hippocampal neuron survival. Very few studies have assessed the effects that human fetal neural progenitor (Skardelly et al., 2011) or NT2N neuron (Philips et al., 2001; Vaysse et al., 2015) transplantation has on reducing lesion volume and/or increasing host neuronal survival. Skardelly et al., found that human fetal progenitor cell transplantation decreased lesion size as measured by T2-weighted MRI at 2.5 months post-injury/cell transplantation and also increased neuronal survival at 3 months post-injury/cell transplantation in the cortex adjacent to the site of injury. However, as only motor related behavioral impairments were measured, not cognition, hippocampal neuron survival was not assessed. Additionally, Philips et al. reported an increase in

hippocampal CA3 pyramidal cell survival after NT2N neuron transplantation. However, this effect was measured only 1 day after cell transplantation; whether this improvement persisted long-term is unknown. Finally, Vaysse et al. recently found a decrease in lesion size and increased tissue sparing after injection of a biomaterial scaffold seeded with hNT2 neurons, but this assessment was by qualitative measures only. Furthermore, while all of these studies demonstrated improvement in some behavioral measures, no analysis of the potential associations between behavioral improvement and decreased lesion volumes or increased neuronal survival after cell transplantation were performed.

To determine if persistent histological changes occur after transplantation of hNSCs into an immunodeficient rat model of TBI, we first assessed lesion volume (as well as spared hippocampal tissue volume and ventricle volume) stereologically. Our results indicate that at 5 months post-transplantation, an approximately 60 mm^3 lesion existed in all ATN TBI animals, regardless of whether given vehicle injection or transplanted with hNSCs. One possibility for why no difference was found in hNSC transplanted animals could have been that the injury itself in our study was too severe and the damaged caused by the initial insult was beyond repair. However, pilot studies in our lab revealed this level of injury was necessary in order for ATNs to develop persistent long-term cognitive deficits. Two other possibilities could be that 1) the delayed nature of our cell transplantation paradigm (hNSCs transplanted 9 days post-injury) did not allow for an early enough intervention to prevent or modulate the initial post-injury wave of damage, and/or that 2) the transplantation of hNSCs into the hemisphere contralateral to injury (as was done in our study) did not allow for the transplanted cells to directly participate in events occurring within or adjacent to the forming lesion.

However, we hypothesized that maximizing transplanted cell survival was of greater importance to behavioral recovery than minimizing lesion size or tissue loss, and previous studies have implicated both transplantation timing as well as location in regards to transplanted cell survival. Analysis of murine NSCs transplanted 2, 7, and 14 days post-TBI demonstrated that cells transplanted at 7 days post-TBI exhibited the highest degree of cell survival (Shear et al., 2011). This finding is in line with previous speculation that 9 days post-injury in the CNS is the optimal time for cell transplantation (Okano et al., 2003; Nakamura and Okano, 2013). However, we have previously compared graft survival of hNSCs transplanted into various rodent models of contusion spinal cord injury at 9, 30 and 60 days post-injury and have found that the timing of transplantation doesn't appear to limit cell survival (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010; Piltti et al., 2013b). However, both the differentiation potential and migration patterns of transplanted hNSCs were affected, suggesting that transplantation timing should be given careful consideration when designing future studies. Transplant location also appears to play a role in cell survival, although definitive conclusions remain controversial. Human fetal neural stem cells, when transplanted into either an injured spinal cord environment compared to uninjured spinal cord (Sontag et al., 2014), or into the injury epicenter compared to locations rostral/caudal to injury (Piltti et al., 2013a), exhibited decreased survival, suggesting that the injury microenvironment may be prohibitive to transplanted cell survival. Conversely, murine NSCs transplanted into the injured hemisphere after mild TBI exhibited increased survival compared to cells transplanted into the contralateral (uninjured) hemisphere (Shear et al., 2011). However, considering the source of the NSCs used in our study (human) and the injury severity (moderate/severe), we chose to

transplant the hNSCs 9 days post-TBI into the hemisphere contralateral to TBI in an attempt to maximize the potential for transplanted cell survival and behavioral recovery. Ultimately, even though no differences were found in either lesion size or hippocampal tissue volume due to hNSC transplantation, cognition was still rescued in hNSC transplanted ATN TBI animals, highlighting that restorative TBI therapies need not necessarily focus primarily on reducing lesion size or tissue loss.

Given then that cognitive improvement was found in the absence of reduced lesion volume or increased hippocampal tissue volume after hNSC transplantation in ATN TBI animals, host hippocampal neuron survival was assessed to determine if differences existed between vehicle and hNSC transplanted animals at the cellular level that could account for the observed behavioral improvement. Previous studies have found that the rodent hippocampus is particularly vulnerable to TBI (Baldwin et al., 1997; McCullers et al., 2002; Anderson et al., 2005; Hall et al., 2005a), and there is longstanding evidence for cognitive deficits in rodents with hippocampal neuronal cell loss (Volpe et al., 1992; Conrad and Roy, 1993). In these studies, lesions to specific hippocampal subregions or surgical procedures that result in region specific hippocampal neuron loss, including neuronal loss in the DG and CA1 regions, caused subsequent memory impairment. Additionally, similar findings have been demonstrated in genetic models of hippocampal neuronal loss (Yamasaki et al., 2007; Myczek et al., 2014), accelerated aging models (Li et al., 2013), models of epilepsy (Silva et al., 2013), early life stress (Brunson et al., 2001), ischemic stroke (Kirino, 1982; Hartman et al., 2005), and TBI (Hicks et al., 1993; Scheff et al., 1997; Scheff et al., 2005; Lu et al., 2007b). Considering that a loss of hippocampal neurons results in cognitive decline, the reverse may be true in that preventing this loss could lead to cognitive improvement. In

our study, we found a significant decrease in hippocampal neurons in both the dentate gyrus (DG, 46% decrease) and cornu ammonis (CA, 62% decrease) zones of ATN TBI animals given vehicle injection that was largely prevented by the transplantation of high passage Shef6 hNSCs. Furthermore, significant positive relationships were found between hippocampal DG and CA number with behavioral improvement on the NPR task, suggesting that a possible link exists between increased hippocampal neuron number and improvement in cognitive function after TBI.

Assessing transplanted hNSC survival, fate, and migration.

To determine the extent to which the transplanted hNSCs survived long-term (5 months) after TBI, we quantified human cell survival stereologically. In this study, high passage (P27) hNSCs were transplanted in addition to low passage (P7) hNSCs in the event that low passage hNSCs were not fully neuralized so as to avoid the potential for non-neural transplanted cell differentiation and teratoma formation (Seminatore et al., 2010). Historically, transplanted human cell survival in immunosufficient animal models of CNS injury and disease (for example, studies using C57Bl6 mice or Sprague Dawley rats) has been shown to be quite poor, even when immunosuppressive drugs were administered (Anderson et al., 2011). In the TBI field to date, only two studies have quantified the number of surviving transplanted human neural cells (Hagan et al., 2003; Wennersten et al., 2006). Both studies reported the terminal amount of surviving human cells to be less than 1% of the original transplant dose. In our study, which utilized immunodeficient ATN rats as the host, we observed greatly improved transplanted human cell survival (9-25%), although not as robust as observed with fetal hNSC transplants into spinal cord injured ATN

rats (Piltti et al., 2013a). Interestingly, we found that high passage hNSCs, which appear to have a greater beneficial effect in facilitating behavioral recovery compared to low passage hNSCs and also resulted in greater host hippocampal neuron survival, survived to a lesser extent than low passage hNSCs (9% vs 25%, respectively). These findings suggest that even though less than 10% of the original dose of high passage hNSCs survived long-term, these cells were still able to exert a promising beneficial effect on cognitive recovery after TBI. However, the question of whether a higher number of cells survived initially and were slowly lost over time, or if only a small amount survived initially and remained throughout the course of the study, still has yet to be answered.

Interestingly, a previous study using this same hNSC cell population demonstrated a neuronal fate preference both *in vitro* and after transplantation into an uninjured immunodeficient host (Haus et al., 2014). Additionally, the transplanted hNSCs exhibited limited migration away from the transplant site. However, in the present study, in response to injury, transplanted hNSCs exhibited a broader differentiation profile and also migrated to a greater extent than in the naïve host. We examined transplanted hNSC fate and found that while the highest percentage of hNSCs still differentiated into NeuN+ neurons (19-38%), astrocyte (13-16%) and oligodendrocyte (11-13%) differentiation readily occurred. Additionally, a significant percentage of cells appeared to remain undifferentiated and continued to express Nestin (10-24%) even after 20 weeks of *in vivo* maturation. These findings suggest that not just one cell type (for example: neurons) may be important for facilitating cognitive recovery after TBI.

3D reconstructions of cell survival demonstrate that many cells migrate away from the transplant site, with some even entering the contralateral (injured) hemisphere and

others migrating to the more ventral aspects of the hippocampus and cortex. A recent study published by Xu et al., reporting for the first time the use of ATN rats in a TBI/cell transplantation model, demonstrated contralateral migration of transplanted hESC-derived OPCs after TBI (Xu et al., 2015), providing further evidence of the migratory potential of human pluripotent-derived stem cells when transplanted after injury. In the clinical setting, transplanted cell migration will likely be a very important consideration. By having a cell population that is migratory, patients will require fewer injections. In our study, we have transplanted cells into 4 different sites (3 total needle tracts) in the hemisphere contralateral to the injury. The location of the transplantation sites were chosen to maximize transplanted cell health (by injecting them distal to the injury site) and also to maximize the potential for transplanted cell migration (by injecting them into the corpus callosum). Our finding that TBI animals receiving hNSC injections in this manner demonstrated improved cognitive function compared to TBI animals receiving vehicle injection supports the effectiveness of this approach. However, even though many of the transplanted hNSCs in our study did exhibit migration away from the injection site, a significant portion remained in adjacent areas, demonstrating the need for further improvements to be made in enhancing the migration of transplanted pluripotent-derived hNSCs.

Conclusions.

In summary, we have shown that hippocampal-dependent spatial memory impairments exist in an immunodeficient ATN model of cortical impact TBI, and that these impairments persist long-term (greater than 2-3 months). Furthermore, we have

demonstrated that in this model of TBI, transplanted hNSCs survive to a greater extent compared to previous TBI xenotransplantation studies utilizing immunosufficient animals given immunosuppressive drugs, and that transplantation of hNSCs restores cognitive function after TBI. Importantly, recovery of cognitive function was achieved without large-scale modifications of lesion or spared tissue volume, suggesting that therapies aimed at improving cognition after brain injury do not necessarily need to prevent tissue loss or reduce lesion volume. Instead, the focus could be centered on modulating the microenvironment within the surviving host tissue to increase, for example, hippocampal neuron survival, which in this study was found to be associated with improved cognitive behavioral recovery. Additionally, some evaluated measures of behavioral recovery, but not all, were improved in this study with the survival of only 9-25% of transplanted hNSCs, suggesting the possibility that further cognitive improvement might be possible if a greater percentage of transplanted human cells survive. Future studies will need to be conducted to evaluate whether increasing the transplantation dose, and thereby potentially increasing the amount of surviving transplanted cells, will lead to further cognitive gains. Additionally, transplantation of different cell types, including fetal- and iPS-derived hNSCs, should also be evaluated in order to determine if an optimal cell population exists to aide in the recovery of cognitive function after TBI.

Conflict of Interest Statement

The authors declare no competing financial interests.

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CHAPTER 4

Ongoing studies of human neural stem cell survival and migration in the traumatic CNS (TBI and SCI) environments

Introduction

The previous chapters in this dissertation have discussed the generation of Xeno-Free human embryonic stem cell (hESC)-derived neural stem cell (hNSC) lines and evaluated their safety in an *in vivo* teratoma formation assay (Chapter 2) and then furthermore tested the efficacy of this hNSC population in ameliorating long-term cognitive dysfunction in a rodent model of traumatic brain injury (TBI) (Chapter 3). In the latter study, the survival of the CD133+/CD34- Shef6 hNSCs at 20 weeks post-transplantation was found to be 9% for high passage Shef6 hNSCs and 25% for low passage hNSCs. Historically, transplanted human cell survival in immunosufficient animal models of CNS injury and disease (for example, studies using C57Bl6 mice or Sprague Dawley rats) has been shown to be quite poor, even when immunosuppressive drugs were administered (Anderson et al., 2011). Additionally, in the TBI field to date, only two studies have quantified the number of surviving transplanted human neural cells (Hagan et al., 2003; Wennersten et al., 2006). Both studies reported the terminal amount of surviving human cells to be less than 1% of the original transplant dose at either 6 days (Hagan et al., 2003) or 6 weeks (Wennersten et al., 2006) post-transplantation. By comparison, in our TBI study, which utilized immunodeficient ATN rats as the host, we observed greatly improved transplanted human cell survival (9-25%) at 20 weeks post-transplantation. However, previous work has demonstrated that between 250-550% of transplanted fetal hNSCs

survive in ATN rats with spinal cord injury (Piltti et al., 2013a). Together, this information begs multiple questions: 1) Do hESC-derived NSCs not survive as well post-transplantation in the TBI niche compared to human fetal-derived NSCs? And if so, is this because hESC-derived NSCs die more readily post-transplantation or do they not proliferate to the same extent post-transplantation as fetal hNSCs? 2) Is overall transplanted hNSC (hESC- or fetal-derived) survival lower in the TBI niche compared to the spinal cord injury (SCI) niche, and is the injured niche a harsher survival environment than the un-injured niche? Finally, 3) Can cell sorting be used to improve transplanted hNSC survival?

To begin to answer some of these questions, I have compiled both published and unpublished results from studies conducted in the laboratories of Drs. Aileen Anderson and Brian Cummings.

Results/Discussion

Transplanted hESC-Derived NSCs Do Not Survive As Well As Fetal hNSCs In The TBI Niche

At 20 weeks post-transplantation, the survival of hESC-derived CD133+/CD34- Shef6 hNSCs was found to be 9% for high passage Shef6 hNSCs and 25% for low passage hNSCs. Interestingly, preliminary unpublished data from my work suggests that fetal hNSCs survive to a greater extent (~70%) in the same TBI/Transplant paradigm as described above for the transplanted Shef6 hNSCs. The fetal hNSCs utilized in these experiments are the same cells from StemCells Inc. as used in the Anderson/Cummings studies of the injured spinal cord. These data suggest that hESC-derived NSCs do not survive as well as human fetal-derived NSCs after transplantation into the TBI microenvironment (summarized in Figure 4.1). However, definitive conclusions are not yet known because cell

survival in only one fetal hNSC transplanted animal has been quantified as the human fetal cell work was unfunded and we did not expend the necessary resources to quantify the human fetal NSCs in the injured TBI niche.

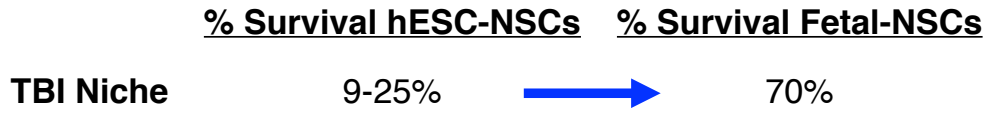

Figure 4.1. The extent of transplanted hNSC survival in the TBI niche depends on cell source. hNSCs derived from human embryonic stem cells (hESCs) survive to a lesser extent (9-25%) than hNSCs derived from human fetal brain tissue (70%).

Additionally, it is unclear if this finding is a result of increased cell death in hESC-derived NSCs compared to fetal hNSCs immediately after the initial transplantation, or decreased proliferation post-transplantation. Preliminary results suggest that possible differences between hESC- and fetal-derived hNSC proliferation may exist post-transplantation. Previous research has shown that fetal-derived hNSCs proliferate at a rate of about 1-3% during the first 2 weeks post-transplantation in the injured spinal cord niche as measured via BrdU incorporation (Sontag et al., 2014). However, our preliminary results of early proliferation of transplanted Shef6 hNSCs suggest that very few to no cells incorporate BrdU either at 2 days or 2 weeks post-transplantation. Thus, my data suggests that very little post-transplantation proliferation of Shef6 hNSCs occur in the TBI niche. The possibility that these differences are a result of differences in the injured niche (TBI vs SCI) must be considered, as no direct comparison of injured niches has yet been performed.

Transplanted hNSCs Survive To A Lesser Extent In The TBI Niche Compared To The SCI Niche

Preliminary unpublished data from our lab (Hal Nguyen) demonstrates that approximately 50-80% of transplanted hESC-derived non-CD133 sorted Shef6 NSCs survive in NOD-SCID mice after SCI. Additionally, between 250-550% of transplanted fetal hNSCs survive in ATN rats with spinal cord injury, depending on whether hNSCs are transplanted into the injury epicenter (250% survival) or in sites rostral/caudal to the injury (550% survival) (Piltti et al., 2013a). More so, unpublished data from our lab (Figure 4.2) confirms the range of survival of fetal hNSCs (hCNS-SC) previously reported in multiple publications (Cummings et al., 2005; Hooshmand et al., 2009; Salazar et al., 2010; Piltti et al., 2013b, a; Sontag et al., 2013; Sontag et al., 2014; Piltti et al., 2015).

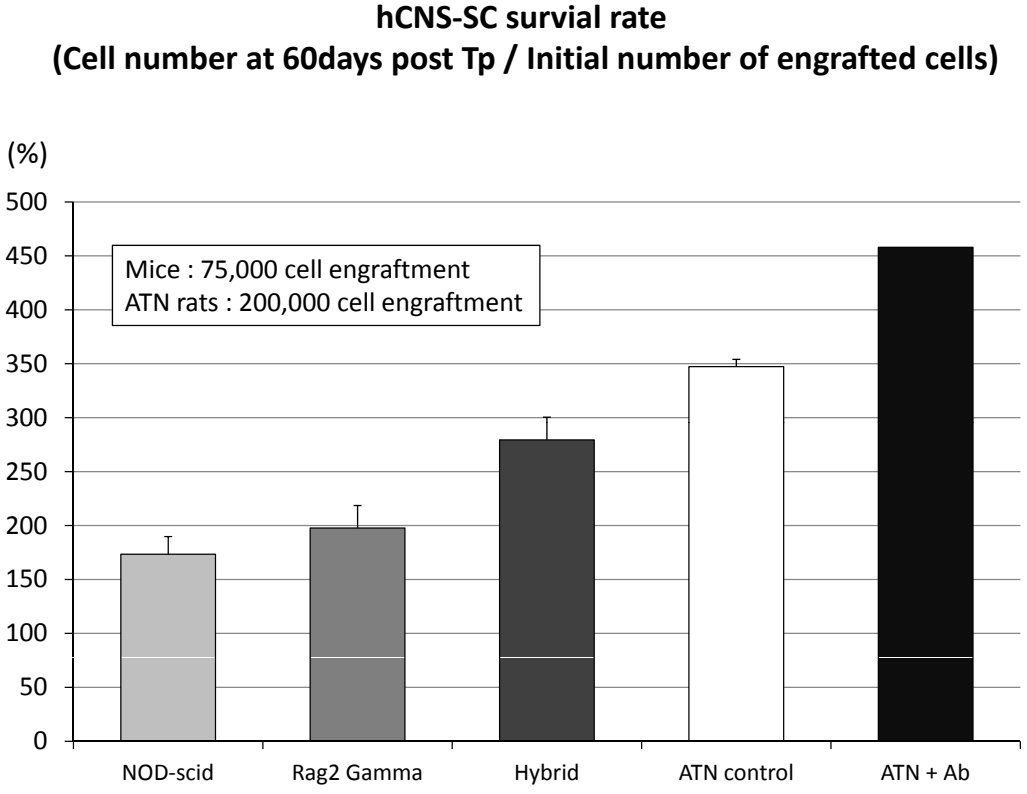

Figure 4.2. The extent of transplanted fetal hNSC (hCNS-SC) survival in the SCI niche varies depending on the immunodeficient rodent species utilized. Fetal hNSCs survive in the SCI niche to a greater extent in immunodeficient rats (ATN control, ATN + Ab) compared to immunodeficient mice (NOD-scid, Rag2 Gamma, Hybrid), though the transplantation dose was scaled up for rats. Within the ATN groups, fetal hNSCs survive to a greater extent when the ATN is given anti-Asialo GM1 (an antibody to deplete Natural Killer (NK) cells). Within the immunodeficient mice groups, transplanted fetal cell survival is greatest in mice bred to have the lowest degree of immunorejection response (Hybrid NOD-scid/ Rag2 Gamma mice) compared to each independent immunodeficient mouse species. Data and graph courtesy of Hirokazu Saiwai.

Conversely, as previously reported (Chapter 3), hESC-derived Shef6 hNSCs survive at a rate of 9-25% in the injured brain, and 70% of fetal-derived hNSCs survive in the injured brain. These data together suggest that hNSCs (either hESC- or fetal-derived) do not survive as well post-transplantation in the TBI niche compared to the SCI niche (summarized in

Figure 4.3, purple arrows). However, one caveat to this conclusion is that the hESC-derived Shef6 hNSCs utilized in the SCI study were not CD133+/CD34- sorted, as they were in the TBI study, so the comparison is not completely direct.

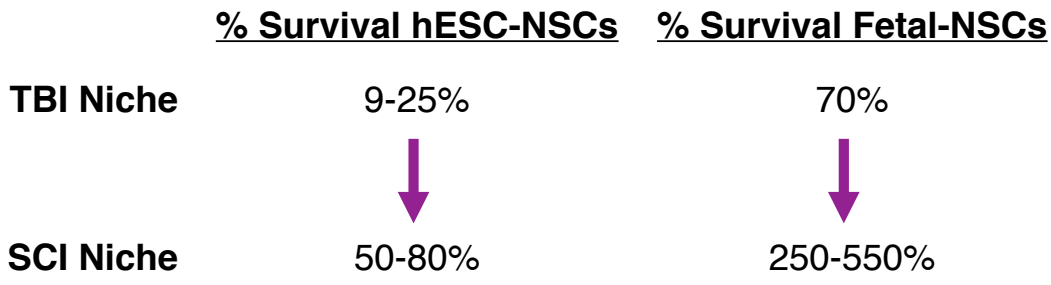

Figure 4.3. The extent of transplanted hESC- and fetal-derived hNSC survival depends on the type of injured transplant niche. hNSCs transplanted into the TBI niche survive to a lesser extent than hNSCs transplanted into the SCI niche, and this difference is consistent in both hESC-derived hNSCs (TBI vs SCI niche, 9-25% vs 50-80%) and fetal-derived hNSCs (TBI vs SCI niche, 70% vs 250-550%). Data on hESC-NSC survival in the SCI niche is courtesy of Hal Nguyen, and data on fetal hNSC survival in the SCI niche is courtesy of Katja Piltti.

The Effect of CD24 Sorting On Transplanted hESC-Derived NSC Survival And Migration After TBI Is Unknown

A small study was undertaken in an attempt to determine if depletion of cells expressing CD24 would influence overall transplanted hNSC survival or migration dynamics. Previously, cell populations expressing CD24 have been shown to be more migratory but less proliferative and form smaller grafts *in vivo* compared to hNSC populations containing few cells expressing CD24 (Pruszak et al., 2009). Our hypothesis, therefore, was that CD24 depletion in our high passage Shef6 hNSCs via magnetic sorting (MACs) would result in greater cell survival post-transplantation (but potentially less

migration). Immediately prior to transplantation, the two hNSC populations (high passage Shef6 hNSCs and CD24 depleted high passage Shef6 hNSCs) were each split into two lots; one for transplantation and one for flow cytometry analysis of CD24 expression. Flow cytometry analysis confirmed that non-depleted high passage Shef6 hNSCs were 62% CD24 positive, while CD24 depleted high passage Shef6 hNSCs were 2% CD24 positive. Both hNSC populations were transplanted (a single hippocampal injection site, delivering 62,500 cells) into ATN rats that had received a TBI (CCI, 2.5 mm depth, 4.5 m/s velocity) nine days earlier. The dose used in this study was identical to a single transplantation site dose (in volume and number of cells) as was used in the studies reported in Chapter 3. The only difference was the additional sorting of the cells, and the single injection site rather than the 4 sites as in Chapter 3 so that we could better track migration from one single site rather then risk the possibly of cells from two sites migrating towards each other. Transplanted animals were analyzed for human cell survival via SC121 immunohistochemistry 8-weeks post-transplantation. However, very minimal human cell survival (1-2 cells per section) was observed in any animal, regardless of hNSC group. This very low cell survival precluded performing full stereological analysis of cell survival.

Prior to transplantation, no cell culture anomalies were observed that could explain the lack of engraftment, nor were there any noted issues during either TBI or transplant surgical procedures for any animal. Additionally, the ATNs utilized in this study were from the same vendor (Charles River/NCI) and were the same vendor stock number (#568) as previously ordered ATNs that exhibited transplanted high passage Shef6 hNSC survival.

There are two obvious explanations for the lack of transplanted hNSC engraftment in this study. First, the overall total number of cells transplanted in this study (62,500) was

lower than in previous studies in which the cells engrafted (250,000). However, the concentration of cells per injection was the same as previous studies (75,000 cells/µl, 833 nl delivered per site). Therefore, it is unlikely that the transplanted cells in the present study did not survive because of a lack of injection site conditioning or paracrine interactions by the transplanted hNSCs. A second possible reason for the lack of engraftment could be that the hNSCs transplanted in this study were from a different frozen vial passage than utilized previously. However, nothing abnormal was noted concerning their growth or appearance prior to transplantation. Finally, other more non-apparent issues may have occurred (such as prolonged disassociation enzyme treatment) that could influence survival but that would normally be accounted for by having multiple injection cohorts and transplanting different cell preparations over multiple days (this was a small study in which all injections were completed in a single day). Additionally, this was a short-term 8 week survival study, not a long-term 20 week study as described previously (Chapter 3). The possibility therefore exists that the limited number of surviving cells at 8 weeks post-transplantation may become more proliferative at a later timepoint that was not able to be observed in this study. In the future, this small study should be repeated, possibly with additional controls including hNSCs from a frozen vial which has demonstrated previous successful engraftment, as well as a long-term survival group so that engraftment can be analyzed at a similar timepoint as assessed in previous studies (20 weeks post-transplantation, Chapter 3).

Conclusions

In summary, these data suggest 1) Transplanted hESC-derived NSCs exhibit decreased

survival post-transplantation in the injured CNS (both brain and spinal cord) compared to human fetal-derived NSCs (Figure 4.4, blue arrows), and 2) The survival of both hESC- and fetal-derived NSCs after transplantation into the TBI microenvironment is lower compared to the SCI microenvironment (Figure 4.4, purple arrows). Additionally, the effect of CD24 depletion on transplanted Shef6 hNSC survival and migration has yet to be determined.

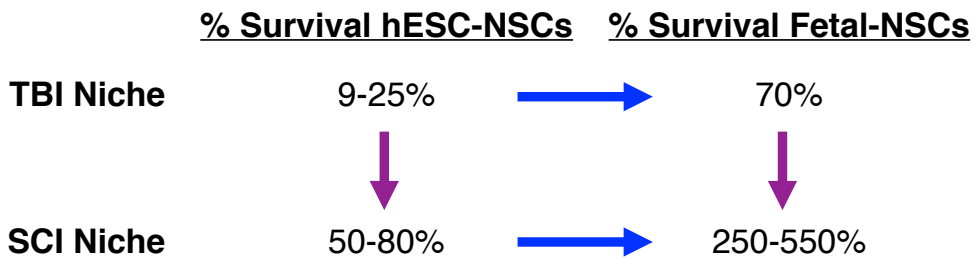

Figure 4.4. The total extent of transplanted hNSC survival depends on both cell source (hESC-derived vs fetal-derived) and the type of injured transplant niche (TBI vs SCI). Summary of transplanted hNSC survival data suggests that hESC-derived hNSCs transplanted into the TBI niche have the lowest survival rate, while fetal-derived hNSCs transplanted into the SCI niche has the highest survival rate. Overall, hESC-derived hNSCs survive to a lower extent than fetal-derived hNSCs, and hNSCs from both sources survive to a lesser extent in the TBI niche compared to the spinal cord niche. Data on hESC-NSC survival in the SCI niche is courtesy of Hal Nguyen, and data on fetal hNSC survival in the SCI niche is courtesy of Katja Piltti.

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CHAPTER 5

Summary and Conclusions

The studies in this dissertation introduce novel methods for the generation of human neural stem cells (hNSCs) from embryonic stem cells with increased clinical relevance. The dissertation then evaluates the therapeutic potential of these cells in promoting cognitive recovery in a rodent model of traumatic brain injury (TBI). Our studies demonstrate for the first time that embryonic-derived hNSCs transplantation improves cognitive performance after TBI and suggests that such an approach warrants further future consideration as a treatment option for TBI and possibly other neurological disorders.

Improving the Clinical Potential of Transplanted Human Neural Stem Cells

In the first set of studies in this dissertation, we developed novel methods for the generation of hNSCs from hESCs in completely Xeno-Free culture conditions (Haus et al., 2014). These methods were developed because stem cell culture systems that utilize animal-derived components have been shown to be more variable than systems using chemically defined products (Koivisto et al., 2004). Additionally, the process of obtaining FDA approval for cell lines cultured with xenogenic components requires additional steps to verify product purity and safety (Ahrlund-Richter et al., 2009). Xeno-Free cell culture aims to remove all non-human, animal-derived products from both the media and substrate and replace them with defined, humanized products, thereby potentially decreasing variability and increasing clinical applicability.

The original hESC lines generated by Thomson et al. (Thomson et al., 1998) utilized standard DMEM cell culture media supplemented with fetal bovine serum (FBS) and were plated on mouse embryonic fibroblasts (MEFs). Subsequent optimization replaced the FBS with a more chemically defined serum replacement (KnockOut Serum Replacement, or KOSR) containing bovine serum albumin (BSA) (Koivisto et al., 2004). Additionally, as research progressed, the use of extracellular membrane (ECM) coating materials such as Matrigel started to become more commonplace, replacing MEFs (Xu et al., 2001; Carpenter et al., 2003). However, both KOSR and Matrigel, although considerably more defined than their predecessors (FBS, MEFs), were still both isolated from individual animal sources and therefore had the potential to exhibit increased lot-to-lot variability. Therefore, in an attempt to eliminate or reduce the potential for lot-to-lot variability to a negligible amount, research groups and companies have continued to develop more chemically defined media formulations (Ludwig and J, 2007).

More recently, as stem cell-based therapies are moving towards, or in some cases into, human clinical trials aimed at repairing the traumatically injured central nervous system (NCT02163876, NCT02302157), there has been increased focus on improving the clinical applicability of stem cell lines so that they are more readily approvable by regulatory agencies. Stem cell therapies utilizing cell lines cultured in the presence of non-human components (such as BSA, or MEFs) have been approved for use in human clinical trials (NCT01217008), however the burden for obtaining approval can be lessened by using cell lines cultured in human only, or Xeno-Free (XF), conditions (Ahrlund-Richter et al., 2009). Multiple XF media formulations have been developed that typically replace BSA with human serum albumin (HSA) and pair media with XF growth substrates such as human

foreskin fibroblasts (HFFs) or XF ECMs such as purified human laminin (LN511) or vitronectin (CELLstart™). Commercially available XF media/substrate combinations include: TeSR2™ with LN511 (STEMCELL Technologies), NutriStem™ XF/FF with LN511 (Biological Industries), RegES™ with hFFs, KO-SR XenoFree™ supplemented with a proprietary growth factor (GF) Cocktail with CELLstart™ (Thermo Fisher Scientific), and Essential 8™ or TESR™-E8™ with Vitronectin (Bergstrom et al., 2011).

In our initial study detailed in this dissertation (see Chapter 2), we developed a novel method for the XF culture of hESCs and subsequent derivation of XF CD133+/CD34- hNSCs (Haus et al., 2014). This protocol utilized KO-SR XenoFree™ media supplemented with a proprietary GF Cocktail and used CELLstart™ as the growth substrate for the XF culture of three hESC lines (Shef3, Shef4, Shef6). Following adaptation of hESCs to XF conditions in a monolayer culture system, two of the three hESC lines were successfully neuralized (and one cell line developed a karyotypic abnormality) initially via a floating EZ-sphere method (Ebert et al., 2013) adapted to XF conditions via the use of X-Vivo 15 media supplemented with N2, B27, EGF, and bFGF. Finally, floating XF neural spheres were supplemented with LIF and plated onto CELLstart™ to form XF, adherent monolayer hNSCs. The XF monolayer hNSC line Shef6 was then magnetically sorted using the MACS system to enrich for the cell surface marker CD133, which has been used to identify stem cell populations within neural tissue (Uchida et al., 2000; Pruszak et al., 2007; Peh et al., 2009), and also to deplete cells expressing CD34, which is expressed on hematopoetic (Civin et al., 1984) and endothelial cells (Fina et al., 1990). The resulting XF CD133+/CD34- Shef6 hNSCs exhibited increased *in vitro* proliferation and neuronal differentiation, and successfully engrafted in immunodeficient NOD-*scid* mice without any evidence for

teratoma formation in long-term (5-month) survival studies. Ultimately, the XF cell culture methods and population enrichment via cell sorting described in Chapter 2 may offer a streamlined approach to generate more readily approvable, expandable, and potentially safer cell populations for CNS transplantation studies.

In the future, XF cell culture will likely continue to evolve to a point in which all steps in the process, from initial pluripotent cell derivation to large-scale bioproduction for clinical use, can be standardized in a manner that produces a stem cell product universally considered safe to use. Indeed, initial work on formalizing standardized, regulatory body approved protocols for the generation and production of human induced pluripotent stem cells (hiPSCs) has recently been introduced and proof of principle clinical grade cell lines produced (Baghbaderani et al., 2015). Additionally, a clinical grade version (Shef6.1) of the Shef6 hESC line utilized in the work described in this dissertation has also been generated (personal communication, Harry Moore, Univ. of Sheffield), and Shef1, an hESC line generated in the same manner as Shef6.1, has been approved by the Medicines and Healthcare Products Regulatory Agency (MHRA), the UK equivalent of the FDA/EMEA, for use in man in a clinical trial for age-related macular degeneration. Thus, the cell lines utilized in the work described in this dissertation have a high degree of clinical applicability.

Evaluating the Efficacy of Human Neural Stem Cell Transplantation in a Rodent Model of TBI

In the second set of studies in this dissertation, we first characterized the long-term cognitive behavioral response of immunodeficient athymic nude (ATN) rats to controlled

cortical impact (CCI) traumatic brain injury (TBI) and then tested whether transplantation of XF CD133+/CD34- Shef6 hNSCs could facilitate cognitive recovery after injury. To date, there are no clinically effective treatment strategies for TBI (Xiong et al., 2009; Maas et al., 2010; Lu et al., 2012). In the past 10 years, over 50 clinical trials in the United States have been completed or terminated to date (October 2015) for drug based TBI therapies (ClinicalTrials.gov). To our knowledge, none of these trials have resulted in the filing of an NDA. There have been 6 phase 1 and/or 2 "stem cell" based clinical trials for the treatment of TBI (October 2015). All have used autologous adult bone marrow mononuclear cells; 4 are based out of The University of Texas Health Science Center, Houston; 1 in India at the Neurogen Brain and Spine Institute; and 1 by SanBio, Inc., in Mountain View, California. While BMMCs may have an immunomodulatory or trophic mechanism of action, there is little evidence these cells reach their CNS target when injected peripherally, and no reliable evidence that these cells have the capacity to differentiate into CNS resident cells. Thus, to date, there are no embryonic stem cell based neural stem cell derivative clinical trials in progress in the United States for TBI.

The preclinical pipeline for cellular therapies of TBI is less definitively summarized. To date, there have been nearly 60 publications using a human based "cell therapy" in a rodent model; more than 70% used amnion, bone marrow, mesenchymal or umbilical cells, not hNSCs with the potential to integrate with the host (Anderson et al., 2011; Gold et al., 2013). While many of these studies have found evidence for behavioral improvement after cell transplantation, the vast majority have not evaluated transplanted cell engraftment and cognitive recovery beyond 1-2 weeks post-injury/cell transplant (Gold et al., 2013). Assessing only early cell survival and behavioral recovery poses two problems: **(1)** If cell

engraftment and subsequent integration is integral to behavioral recovery, evaluation at later time points becomes critical as transplanted human cells will likely require at a minimum several months to fully integrate and differentiate into mature neural cell types, and **(2)** If long-term behavioral improvement is the desired clinical outcome, evaluation of recovery within days to weeks of injury or treatment in preclinical rodent studies is not informative for long-term behavioral gains in future human clinical studies. Therefore, in Chapter 3 of this dissertation we have addressed these shortcomings in the TBI and stem cell transplantation literature by developing a novel TBI xenotransplantation model that utilizes immunodeficient athymic nude (ATN) rats as the host recipient for the post-TBI transplantation of human embryonic stem cell (hESC) derived NSCs and have furthermore evaluated cognition in these animals at long-term (≥2 months) time points post-TBI/cell transplantation.

To summarize our findings from Chapter 3, we have shown that hippocampal-dependent spatial memory impairments exist in immunodeficient ATN rats after TBI, and that these impairments persist long-term (greater than 2-3 months). Furthermore, we have demonstrated that in this model of TBI, transplanted hNSCs survive to a greater extent compared to previous TBI xenotransplantation studies utilizing immunosufficient animals given immunosuppressive drugs. Finally, we show that transplantation of hNSCs restores cognitive function after TBI and that recovery of cognitive function was associated with increased host hippocampal neuron survival.

After TBI, rodents often exhibit cognitive impairment that can last up to several months post-injury, and stem cell transplantation has, in some cases, been shown to facilitate cognitive recovery. By limiting our discussion to transplanted cells derived from

the human CNS or from human stem cells differentiated towards a CNS fate, we find that previous studies (Table 5.1) have shown efficacy for the transplantation of fetal human neural stem/progenitor cells (Hagan et al., 2003; Al Nimer et al., 2004; Wennersten et al., 2004; Gao et al., 2006; Wennersten et al., 2006; Skardelly et al., 2011; Lundberg et al., 2012; Poltavtseva et al., 2012; Wang et al., 2012; Hwang do et al., 2014) as well as human NT2N neurons (Muir et al., 1999; Philips et al., 2001; Watson et al., 2003; Longhi et al., 2004; Zhang et al., 2005; Vaysse et al., 2015). Additionally, recent studies have reported the successful engraftment and survival of human pluripotent-derived stem cells in rodent models of TBI, including the demonstration of human induced pluripotent stem cell (hiPSC) derived NSC engraftment and cell tracking (Tang et al., 2013) as well as the first demonstration of successful hESC-derived oligodendrocyte progenitor cell (OPC) engraftment in an ATN TBI model (Xu et al., 2015). Critically however, only one of these studies evaluated cognitive function long-term (2 or more months) after injury/ transplantation and reported improvement on a cognitive measure (Skardelly et al., 2011), and to our knowledge, zero studies to date have been conducted assessing the effects of hESC-NSC transplantation in modulating cognitive function after TBI. Our study therefore is the first demonstration of hESC-NSC transplant-mediated recovery of cognitive function post-TBI.

Table 5.1. Human neural cell therapy studies in rodent models of traumatic brain injury.

(Author, year)	Cell Type	Host Species	Final Time Point (post-transplant)	Cell Survival	Behavioral Assessment
(Al Nimer et al., 2004)	Fetal hNSC	Sprague-Dawley	6 Weeks	N.R.	N.A.
(Gao et al., 2006)	Fetal hNSC	Sprague-Dawley	2 Weeks	N.R.	Improved MWM
(Longhi et al., 2004)	NT2N Neurons	C57BL/6	4 Weeks	N.R.	Improved MWM
(Muir et al., 1999)	hNT Neurons	Sprague-Dawley	2 Weeks	N.R.	No Differences
(Philips et al., 2001)	NT2N Neurons	Sprague-Dawley	2 and 4 Weeks	N.R.	No Differences
(Skardelly et al., 2011)	Fetal hNPC	Sprague-Dawley	12 Weeks	N.R.	Improved Rotarod, mNSS

Table 5.1. Human neural cell therapy studies in rodent models of traumatic brain injury.

(Author, year)	Cell Type	Host Species	Final Time Point (post-transplant)	Cell Survival	Behavioral Assessment
(Wang et al., 2012)	Fetal hNSC	Sprague-Dawley	4 Days	N.R.	N.A.
(Wennersten et al., 2004)	Fetal hNSC	Sprague-Dawley	2 and 6 Weeks	N.R.	N.A.
(Watson et al., 2003)	NT2N Neurons	C57BL/6	4 Weeks	N.R.	Improved MWM
(Zhang et al., 2005)	NT2N Neurons	Sprague-Dawley	4, 8, and 12 Weeks	N.R.	No Differences
(Poltavtseva et al., 2012)	Fetal hMSC and hNSC	Outbred Albino Rat	3 Weeks	N.R.	Improved Limb Function
(Vaysse et al., 2015)	hNT2 Neurons	Sprague-Dawley	3 Months	N.R.	Improved Grip Strength
(Hwang do et al., 2014)	Fetal hNSC	Sprague-Dawley	14 Days	N.R.	N.A.
(Tang et al., 2013)	hiPSC-NSC	Sprague-Dawley	30 Days	N.R.	Improved Rotarod, mNSS
(Lundberg et al., 2012)	Fetal hNSC	Sprague-Dawley	1 Day	N.R.	N.A.
(Xu et al., 2015)	hESC-OPC	Athymic Nude Rat	3 Months	N.R.	N.A.
(Wennersten et al., 2006)	Fetal hNSC	Sprague-Dawley	3 or 6 Weeks, 6 Months	0.03-0.2%	N.A.

A total of 18 papers have transplanted human stem cells of a neural fate into a rodent model of traumatic brain injury. Only two of these papers reported a quantified cell survival, and only one of them used an immunodeficient rodent strain (though, cell survival quantification was not performed in this study). Author, transplanted human stem cell population, host species, length of study, quantified cell survival and functional assessments in treated compared with untreated animals is shown.
hNSC: Human neural stem cell; mNSS: Modified neurological severity score; MWM: Morris water maze; N.R.: Not reported; N.A.: Not assessed

While we did not detect any histological differences in lesion volume, total spared hippocampal tissue volume, or ventricle volume, we did demonstrate significant host neuronal loss after TBI that was prevented by XF CD133+/CD34- Shef6 hNSC transplantation. Previous studies have found that the rodent hippocampus is particularly vulnerable to cortical impact TBI (Baldwin et al., 1997; McCullers et al., 2002; Anderson et al., 2005; Hall et al., 2005a), and there is longstanding evidence for cognitive deficits in rodents with hippocampal neuronal cell loss (Volpe et al., 1992; Conrad and Roy, 1993), particularly in hippocampal subregions such as the Dentate Gyrus (DG) and CA1, and similar findings of cognitive decline have been demonstrated after TBI (Hicks et al., 1993; Scheff et al., 1997; Scheff et al., 2005; Lu et al., 2007b) and in genetic models of hippocampal neuronal loss (Yamasaki et al., 2007; Myczek et al., 2014).

Considering that a loss of hippocampal neurons results in cognitive decline, the reverse may be true - that preventing this loss could lead to cognitive improvement (or prevention of cognitive loss post-TBI). In our study, we found a significant decrease in host hippocampal neurons in both the DG and CA regions of ATN TBI animals given vehicle injection that was largely prevented by the transplantation of high passage Shef6 hNSCs. Furthermore, we found significant positive relationships between hippocampal DG and CA number with behavioral improvement on the NPR task. These results suggest that a possible link exists between increased hippocampal neuron number in hNSC transplanted animals and improvement in cognitive function after TBI.

Improving the Survival and Migration of Transplanted Human Neural Stem Cells

We also quantified human cell survival in TBI transplanted ATN rats stereologically and found that at 20 weeks post-transplantation, depending on the cell line, transplanted hNSCs exhibited between 9% (high passage CD133+/CD34 Shef6 hNSCs) and 25% (low passage CD133+/CD34 Shef6 hNSCs) survival relative to the total number of transplanted cells. Historically, transplanted human cell survival in immunosufficient animal models of CNS injury and disease (for example, studies using C57Bl6 mice or Sprague Dawley rats) has been shown to be quite poor, even when immunosuppressive drugs were administered (Anderson et al., 2011). In the TBI field to date, only two studies have quantified the number of surviving transplanted human neural cells (Hagan et al., 2003; Wennersten et al., 2006) Both studies reported the terminal amount of surviving human cells to be less than 1% of the original transplant dose. By comparison, in our TBI study, which utilized

immunodeficient ATN rats as the host, we observed greatly improved transplanted human cell survival (9-25%).

Interestingly, very preliminary data from our lab (Daniel Haus, unpublished) suggests that fetal hNSCs survive to a greater extent (~70%; based on quantification of 1 animal) in the same TBI/Transplant paradigm as described above for the transplanted Shef6 hNSCs. These data suggest that hESC-derived NSCs do not survive as well as human fetal-derived NSCs after transplantation into the TBI microenvironment. However, it is unclear if this finding is a result of increased cell death in hESC-derived NSCs compared to fetal hNSCs or decreased proliferation post-transplantation. Furthermore, the survival of transplanted hNSCs after TBI also appears to be lower than the survival of transplanted hNSCs post-SCI. Preliminary unpublished data from our lab (Hal Nguyen) demonstrates that approximately 50-80% of transplanted hESC-derived non-CD133 sorted Shef6 hNSCs survive in NOD-SCID mice after SCI. Additionally, up to 250-550% of transplanted fetal hNSCs survive in ATN rats with spinal cord injury (Piltti et al., 2013a). Overall, these data suggest **(1)** Transplanted hESC-derived NSCs exhibit decreased survival post-transplantation in the injured CNS (both brain and spinal cord) compared to human fetal-derived NSCs, and **(2)** The survival of both hESC- and fetal-derived NSCs after transplantation into the TBI microenvironment is lower compared to the SCI microenvironment. Importantly, however, our findings also suggest that even though less than 10% of the original dose of high passage CD133+/CD34- Shef6 hNSCs survived long-term after transplantation in our model of TBI, these cells were still able to exert a promising beneficial effect on cognitive recovery.

Another key factor in developing an effective stem cell transplant-based therapy for TBI, beyond achieving high levels of transplanted cell engraftment and survival, is ensuring

that the transplanted cells can migrate away from the transplant site and reach their intended target. Previous studies have shown that transplanted human neural cells, and in particular those derived from pluripotent embryonic stem cells and transplanted into the brain, tend to form dense clusters with limited (less than 1-2mm) outward cell migration (Fricker et al., 1999; Tabar et al., 2005; Roy et al., 2006). The rationale for this limited migration has been suggested to be related to the mismatch between host tissue and transplant tissue maturation, lack of guiding cues in the adult host (Svendsen and Caldwell, 2000), glial scarring at the site of injection (Reier et al., 1983; Kinouchi et al., 2003), and graft-intrinsic interactions between transplanted NSCs and their neuronal progeny (Ladewig et al., 2014). Conversely, human fetal neural stem cells transplanted into rats with spinal cord injury have been shown to migrate distances greater than 10mm (Piltti et al., 2013b).

In our study, we observed that many engrafted CD133+/CD34 Shef6 hNSCs had migrated away from the transplant site, with some even entering the contralateral (injured) hemisphere while others had migrated to the more ventral aspects of the hippocampus and cortex. Because our transplantation paradigm utilized four different injection sites, an accurate estimation of maximal transplanted cell migration is difficult to obtain. However, estimating the distance from human cells found in the contralateral hemisphere to what would be the nearest possible injection site would suggest that engrafted CD133+/CD34 Shef6 hNSCs had migrated at least 3mm by 20 weeks post-transplantation. Additionally, a recent study published by Xu et al. demonstrated contralateral migration of at least 5mm of transplanted hESC-derived OPCs after TBI (Xu et al., 2015). Such studies provide evidence of the migratory potential of human pluripotent-derived stem cells when transplanted after

injury. However, even though many of the transplanted hNSCs in our study did exhibit migration away from the injection site, a significant portion remained in adjacent areas, demonstrating the need for further improvements to be made in enhancing the migration of transplanted pluripotent-derived hNSCs. Additionally, the question of whether the increased migration of fetal hNSCs transplanted after SCI compared to embryonic-derived hNSCs transplanted after TBI is due to cell type (fetal vs. embryonic-derived) or transplant niche (spinal cord vs. brain) has yet to be directly examined. Towards this end, preliminary unpublished data from our lab (Hal Nguyen) demonstrates that transplanted embryonic-derived hNSCs migrate up to 8mm after SCI, which suggests that the injured spinal cord niche may be more amenable to transplanted cell migration than the injured brain. However, one caveat to this work was that it was performed in NOD-SCID mice, not ATN rats, and therefore mouse vs. rat differences may also be a factor.

Concluding Remarks and Future Recommendations

Overall, stem cell transplantation should be viewed as a promising approach for treating TBI-related cognitive impairment. Several clinical trials utilizing human mesenchymal stem cells to treat patients with TBI have been approved and are upcoming (NCT02525432, NCT02416492), ongoing (NCT02028104, NCT01851083) or have been completed (NCT00254722, NCT01575470). All of these trials have or will utilize adult bone marrow-derived cells with endpoints including cell and surgical procedure safety, reduced neurological events (seizures, adverse changes in Glasgow Coma Score), improved macro and micro structural properties of grey matter and white matter regions visualized using high-resolution anatomical MRI and diffusion tensor imaging, and improved motor and gait

performance as measured by the Fugl-Meyer Motor Scale (FMMS) and the Disability Rating Scale (DRS). To date, however, none of the completed TBI/stem cell trials have reported any results. Additionally, a Phase 1/2 clinical trial sponsored by Asterias Biotherapeutics for cervical spinal cord injury using hESC-derived oligodendrocyte progenitor cells (AST-OPC1) has also recently gained approval (NCT02302157). Together, these studies have paved the way for stem cell therapies for patients with TBI using differentiated cells derived originally from hESCs. In the studies described in this dissertation, we have developed a novel hESC-derived CD133+/CD34- hNSC population and demonstrated efficacy in restoring cognition in rodents after TBI. However, there are still many unknowns to consider before embarking on the possibility of clinical trials using the cell therapy described in Chapters 2, 3, and 4 of this dissertation. These unknowns include:

(1) How can we improve the survival of transplanted hNSCs? In studies utilizing immunosufficient host animals given immunosuppression, likely a large number of transplanted cells are lost due to immunorejection (Anderson et al., 2011). However, in our study from Chapter 3, immunodeficient ATN rats were used and transplanted cell survival was still only 9-25%. One possible approach to further increase transplanted cell survival could be to transplant cells in combination with scaffold or hydrogel biomaterials that would soften the initial impact of injection and could also provide pro-survival trophic support to the stem cells. A second possible approach could be to utilize cell sorting approaches to either enrich for cells with a greater potential for survival or deplete cells with low survival potential (for example, cells expressing CD24) prior to transplantation.

(2) Our data demonstrates demonstrates that at 20 weeks post-transplantation, 9-25% of

the transplanted CD133+/CD34- Shef6 hNSCs survive in the TBI niche. However, the early survival and proliferation dynamics of these cells have yet to be fully analyzed. While preliminary results suggest that transplanted hESC-derived hNSCs do not survive as well as fetal-derived hNSCs in the TBI niche, and furthermore that hESC- or fetal-derived hNSC survival is lower in the TBI niche compared to the SCI niche, direct comparison studies have yet to be undertaken.

(3) While transplantation of CD133+/CD34- Shef6 hNSCs improved cognition after TBI in our study, the question of whether recovery was due to transplanted cell integration, transplant-secreted trophic support, or some other mechanism is not known. Answering this question will inform us as to whether long-term transplanted cell survival is needed, or if cell transplantation itself is even necessary. Future studies will need to be conducted to either turn off or selectively kill transplanted cells, or to transplant cells that are defective in making specific growth factors.

(4) Finally, transplantation of different cell types, including fetal- and iPS-derived hNSCs, should also be evaluated in order to determine if an optimal cell population exists to aide in the recovery of cognitive function after TBI.

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