UNIVERSITY OF CALIFORNIA SAN DIEGO

Neural Stem Cell Transplantation for Spinal Cord Injury: Differentiation, Migration, Integration, and Safety of Xenograft-Derived Astrocytes

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by

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The Thesis of Brian Van Lien is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

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DEDICATION

I would like to dedicate my thesis to my father, Huy Quoc Lien, and mother, Co Hoang Tran.

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ABSTRACT OF THE THESIS

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Neural stem cells (NSCs) can differentiate into both neurons and glia after transplantation into sites of spinal cord injury (SCI). The neuronal component of stem cell grafts has the potential to form functional synaptic relays across the lesion site. The glial component, on the other hand, may reform a blood–spinal cord barrier (BSCB),

support neuronal function, and potentially contribute to remyelination. We performed a long-term, time-course study for 1.5 years focused on astrocyte survival, migration, differentiation, integration, and safety following transplantation of human NSCs into sites of C5 hemisection in immunodeficient rats. Subjects were sacrificed at 1, 3, 6, 12, and 18 months after transplantation.

Notably, NSCs that adopted a neuronal fate did not migrate from the lesion site. In contrast, transplanted cells that adopted astrocyte fates exhibited long-distance migration from the lesion site and through host white matter in rostrocaudal directions. NSCs migrated from the lesion slowly, at a mean rate of 2-3 mm per month, divided as they migrated, and gradually differentiated into astrocytes. After 1.5 years, astrocytes migrated 9 spinal cord segments, caudally to the mid-thoracic level, and rostrally into the brainstem. The migrated human astrocytes joined the endogenous population of astrocytes in the host spinal cord, extended endfeet towards cellular constituents of the blood-spinal cord barrier, and formed perivascular astrocytic networks connected by gap junctions, suggesting structural and potentially functional integration. No adverse consequences of this extended glial migration were detected. Thus, human astrocytes can migrate from NSC grafts transplanted into site of SCI and safely integrate into the host central nervous system.

Chapter 1: Introduction

Spinal Cord Injury and Pathophysiology. For millennia, physicians have observed that the spinal cord does not functionally regenerate after injury (Sofroniew et al., 2018). Today in the United States, approximately 250,000 people live with a spinal cord injury (SCI) and 11,000 people suffer from new injuries annually (Silva et al., 2014). The most common cause of SCI is trauma, specifically due to vehicular accidents (38%), acts of violence (26%), falls (22%), and sports accidents (7%) (Dobkin and Havton, 2004). Rarer causes of SCI involve tumors, ischemia, or infections of the spinal cord (Dobkin and Havton, 2004). Damage to ascending sensory tracts and descending motor tracts in the spinal cord causes motor, sensory, and autonomic deficits that lead to diminished quality of life (Lu, 2017; Silva et al., 2014). SCI can lead to paraplegia, which is impairment of sensorimotor function in the lower extremities, or quadriplegia, which is impairment of sensorimotor function in the upper and lower extremities. Current treatments for SCI are mostly palliative and none are curative; thus, there is an urgent need for the development of novel therapies to treat SCI (Silva et al., 2014).

The pathophysiology of SCI consists of the primary injury mechanism and the secondary injury mechanism. The primary injury is the result of laceration, contusion, or infarction of the spinal cord leading to several neurological insults, including severed axons, blood-spinal cord barrier (BSCB) breakdown, and cellular damage to neurons, astrocytes, and oligodendrocytes (Silva et al., 2014; Dobkin and Havton, 2004; Lee-Liu et al., 2013). The primary injury leads to a cascade of biological events that characterize the secondary injury, which causes further damage to spinal cord tissue and further loss of function (Silva et al., 2014; Dobkin and Havton, 2004). The consequences of

secondary injury include demyelination, ischemia, and necrosis of cells in the spinal cord (Dobkin and Havton, 2004). For these reasons, the design of future treatments for SCI should attempt to minimize the extent of secondary injury.

Unlike mammals, other vertebrates such as fish and amphibians have spinal cords that can functionally regenerate after injury (Lee-Liu et al., 2013; Richardson et al., 1980). A major hurdle to functional recovery in humans after SCI is the failure of axons in the adult mammalian central nervous system (CNS) to spontaneously regenerate after injury (He and Jin, 2016). In the adult mammalian nervous system, only axons in the peripheral nervous system (PNS) can regenerate (Richardson et al., 1980; Lu and Tuszynski, 2008; Huebner and Strittmatter, 2009). Aguayo and colleagues were first to show that a permissive PNS graft allows adult CNS axons to regenerate, leading to the discovery that the PNS environment is stimulatory for regeneration, and the CNS environment is inhibitory for regeneration (Richardson et al., 1980; Huebner and Strittmatter, 2009; He and Jin, 2016). It is now well established that adult mammalian CNS axons fail to regenerate due to a combination of extrinsic and intrinsic influences (Richardson et al., 1980; Lu and Tuszynski, 2008; van Niekerk et al., 2016; He and Jin, 2016).

Chondroitin sulfate proteoglycans (CSPGs), present in the extracellular environment of the mature CNS, are thought by many to inhibit axon regeneration (Bradbury et al., 2002; Bradbury and Carter, 2011; van Niekerk et al., 2016). After SCI, it has been proposed that reactive astrocytes contribute to inhibiting axon regeneration by formation of a “glial scar,” a complex pathophysiological response that creates a physical barrier for regeneration and increases the expression and secretion of CSPGs

by reactive astrocytes (Bradbury et al., 2002; Silva et al., 2014). Other cells besides reactive astrocytes, such as microglia, macrophages, pericytes, and fibroblasts also release CSPGs (van Niekerk et al., 2016; Liddelow and Barres, 2016). The use of the enzyme chondroitinase ABC (ChABC) has been shown to promote axon regeneration and functional recovery after SCI by degrading chondroitin sulfate glycosaminoglycan (CS-GAG) chains from CSPGs (Bradbury et al., 2002; Bradbury and Carter, 2011; van Niekerk et al., 2016). However, recent data published by Sofroniew and colleagues suggest that the “glial scar” promotes regeneration of the spinal cord after injury, challenging the widespread view that the “glial scar” serves as a barrier to CNS repair (Anderson et al., 2016). There is emerging evidence that specific types of CSPGs, such as CSPG4 and CSPG5, can support regeneration of CNS axons (Liddelow and Barres, 2016; Anderson et al., 2016). Overall, these provocative results paint a more complicated picture of the influence of the CSPGs on CNS axon regeneration, suggesting that not all CSPGs are inhibitory as previously believed. Future studies are surely required to clarify whether certain CSPGs subtypes are growth-promoting or growth-inhibitory in order to develop meaningful therapies for SCI.

Many molecules in the extrinsic environment that inhibit CNS axon regeneration, such as myelin associated inhibitors (MAIs), have been identified in the decades since Aguayo and colleagues first discovered that the mature CNS environment is inhibitory to axon regeneration (Huebner and Strittmatter, 2009). Oligodendrocytes, the myelinating glial cell in the CNS, secrete MAIs that prevent axonal outgrowth, such as Nogo-A, myelin associated glycoproteins (MAG), oligodendrocyte myelin glycoprotein (OMgp), ephrin B-3, and Semaphron 4D (Sema4D) (Silva et al., 2014). In contrast, Schwann

cells, the myelinating glial cell in the PNS, rarely secrete MAIs (Silva et al., 2014). In fact, Schwann cells support functional axon regeneration by secreting neurotrophic factors that promote neuronal survival and increasing protein synthesis in injured axons through polyribosome transfer (van Niekerk et al., 2016).

In addition to inhibitory molecules in the mature CNS environment that block axon regeneration, there is also a lack of diffusible neurotrophic factors to promote axon regeneration (van Niekerk et al., 2016). PNS axon regeneration is enabled by the secretion of neurotrophic factors in gradients that guide regenerating axons through the mature PNS environment and promote neuronal survival through trophic support (van Niekerk et al., 2016). CNS axons have been shown to regenerate when neurotrophic factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), or brain-derived neurotrophic factor (BDNF) are provided in gradients *in vivo* (van Niekerk et al., 2016). It is important to note, however, that neurotropic factors are typically not sufficient to elicit regeneration of corticospinal tract (CST) axons (van Niekerk et al., 2016), which form critical circuits involved in voluntary movement. Although CST axons, compared to other axons in the motor system, tend to be refractory towards regeneration, success has been found by providing a permissive environment for axon regeneration using caudalized neural stem cell grafts (Kadoya et al., 2016) or even by manipulating growth promoting signaling pathways, such as through conditional deletion of the gene, phosphatase and tensin homolog (PTEN) (Liu et al, 2010).

Adult mammalian CNS neurons are not only inhibited by the extrinsic environment of the mature CNS, but also lack the intrinsic capacity for growth. The intrinsic growth state of injured CNS neurons can be modulated by manipulation of a

variety of signaling pathways, such as the mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 3 (STAT3), Kruppel-like family of transcription factors (KLFs), b-RAF, and Sox11 pathways (He and Jin, 2016). The most robust examples of CNS axon regeneration have been observed after co-deletion of PTEN and suppressor of cytokine signaling 3 (SOCS3) or by deletion of either gene alone (Park et al., 2008; Smith et al., 2009; Liu et al., 2010; Sun et al., 2011). Aside from differences in activation of signaling pathways required for axon regeneration, CNS axons also form dystrophic endings after injury that fail to initiate the growth cones necessary for successful regeneration (van Niekerk et al., 2016). Unlike CNS axons, PNS axons form growth cones containing a stabilized, as opposed to disorganized, microtubule network (van Niekerk et al., 2016). In addition, the lack of localization of ribosomes and mRNA to the axon tip may also contribute to the neuron intrinsic failure for CNS axon regeneration after injury (van Niekerk et al., 2016).

Neural Stem Cells. One practical strategy to restore function after SCI may come in the form of cell replacement therapies. Lu and colleagues have developed a method of stem cell transplantation by embedding neural stem cells into fibrin/thrombin matrices containing multiple growth factors prior to grafting into the injured host spinal cord (Lu et al., 2012). This method provides a permissive environment for regeneration, enabling injured host axons to regenerate into the graft (Lu et al., 2012). In addition, this method allows neural stem cells to survive in the inhibitory adult CNS environment, extend axons long distances that form synapses with host cells, and support functional motor recovery (Lu et al., 2012). Many follow-up studies since suggest that transplantation of

NSCs may lead to functional recovery due to formation of relay circuits, neuroprotection, and remyelination (Lu, 2017).

When transplanted into sites of spinal cord injury, neural stem cells (NSCs) differentiate into neurons and glia (Lu, 2017; Kadoya et al., 2016; Lu et al., 2017; Rosenzweig et al., 2018). The neuronal component of stem cell grafts extend axons long distances in the inhibitory environment of the mature CNS and form functional synaptic relays across sites of SCI (Lu et al., 2012; Lu et al., 2017; Rosenzweig et al., 2018). The glial component of NSC grafts may have a variety of therapeutic benefits for cell replacement, especially since SCI can cause loss of oligodendrocytes required to myelinate axons, as well as loss of astrocytes required to support neuronal function and form the selectively permeable BSCB to maintain CNS homeostasis (Goldman, 2017; Matsushita et al., 2015; Barres, 2008).

Astrocytes and the Blood-Brain Barrier. Named after their star-like morphology, astrocytes are a heterogeneous group of cells known to play critical and dynamic roles in the CNS (Zhang and Barres, 2010; Khakh and Sofroniew, 2015). Astrocytes in the CNS participate in the BBB and BSCB, induce synapse formation, modulate synaptic transmission and plasticity, secrete neurotrophic factors to promote neuronal survival, release gliotransmitters to communicate with neurons, and regulate extracellular levels of ions and neurotransmitters (Zhang and Barres, 2010; Barres, 2008; Abbott et al, 2006). Although astrocytes are implicated in almost every disorder of the CNS, the functional role of these important cells have received much less attention in comparison to neurons and remain poorly understood in the fields of neuroscience and medicine (Zhang and Barres, 2010).

In response to CNS injury in mammals, reactive astrocytes become activated, proliferate, and undergo hypertrophy in a heterogeneous secondary response called reactive astrogliosis; these reactive astrocytes, depending on the subtype and type of injury, appear to either have neurotoxic or neuroprotective effects (Liddelow and Barres, 2016; Liddelow, et al., 2017; Khakh and Sofroniew, 2015). The complex “glial scar” that forms after neurological injury is composed of these reactive astrocytes, as well as non-neural cells such as pericytes and meningeal cells, and glial cells such as reactive oligodendrocyte precursor cells and reactive microglia (Sofroniew et al., 2018; Moeendarbary et al., 2017; Liddelow and Barres, 2017). Evidence suggests that the “glial scar” is involved in sealing the injury site, limiting spread of inflammation, re-establishing the BSCB, and possibly blocking axon outgrowth (Moeendarbary et al., 2017; Sofroniew et al., 2018). The secondary events that characterize reactive astrogliosis appear to depend on the type of CNS injury, such as trauma, inflammation, or ischemia (Khakh and Sofroniew, 2015). The sources of newly proliferated reactive astrocytes after SCI include both the local population of perivascular astrocytes that make up the BSCB and a migrated population of glial progenitors from the ependyma, the membrane of glial cells lining the cerebral spinal fluid (CSF)-filled central canal of the spinal cord (Khakh and Sofroniew, 2015; Barnabé-Heider et al., 2010). Ablation of reactive astrocytes prevents the BSCB barrier from reforming after injury, leading to increased inflammation at the injury site, demyelination, neuronal degeneration in the spinal cord, and impaired motor function, highlighting the protective role of reactive astrocytes after SCI (Faulkner et al., 2004).

Astrocytes are also believed to play an important role in induction, maintenance, and reestablishment of the BBB and BSCB during development, throughout adulthood, and after injury (Abbott et al., 2006; Barres 2008; Sofroniew and Vinters, 2010). The BBB and BSCB are functionally equivalent in that both are selectively permeable barriers formed by tight junctions between endothelial cells lining the microvessels that distribute blood to the brain and the spinal cord (Abbott et al., 2006; Bartanusz et al., 2011). In the BBB and BSCB, capillary endothelial cells are surrounded by a layer of extracellular matrix called the basal lamina, contractile cells of mesodermal origin called pericytes, and glial processes called astrocytic perivascular endfeet (Abbott et al., 2006). BBB and BSCB phenotype can be induced by factors released by astrocytes, including transforming growth factor-β (TGFβ), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and angiopoetin 1 (Ang-1) (Abbott et al., 2006). Factors expressed by astrocytic perivascular endfeet, such as Ang-1 and src-suppressed C-kinase substrate (SSeCKS), have been shown to increase the integrity of the BSCB by upregulating tight junctions between endothelial cells (Lee et al., 2003).

After traumatic injury, damage to the BBB and BSCB leads to loss of astrocyte-endothelial cell interactions, which can disturb CNS homeostasis and exacerbate neuropathology (Abbott et al., 2006, Faulkner et al., 2004). Specifically, breakdown of the BBB and BSCB can lead to the leakage of proteins into the CNS, leading to vasogenic edema formation that can last for weeks and cause swelling that damages axons, neurons, myelin, and oligodendrocytes (Kimura et al., 2010). Astrocytic perivascular endfeet, a major component of the BBB and BSCB, are known to express aquaporin water channels, which are involved in clearance of excess water from the

CNS (Kimura et al., 2010). After SCI, damage to astrocytic perivascular endfeet leads to swelling that causes secondary demyelination and neuronal death (Kimura et al., 2010; Sharma, 2005). SCI in humans and animal models nearly universally causes primary injury to the BSCB that can persist for months before being fully repaired, further contributing to secondary injury of previous unaffected areas of the spinal cord near the site of injury (Matsushita et al., 2015; Maikos and Shreiber, 2007; Sharma, 2005). There is evidence suggesting that stem cells may be therapeutically useful in decreasing the effects of secondary injury and improving motor function (Matsushita et al., 2015; Eve et al., 2018). Thus, the role of astrocytes in the BSCB should carefully considered when designing and implementing clinical trials involving the use of stem cells for SCI.

Research objectives. The goal of my thesis was to research the safety of a preclinical cell transplantation therapy developed in our laboratory to treat SCI. This therapy involves grafting human NSCs combined with a fibrin matrix and growth factor cocktail into the site of SCI. For my thesis, I focused on the survival, migration, differentiation, and integration of the glial component of NSC grafts. Here, I report extensive astroglial migration after transplantion of human NSCs into spinal cord hemisection sites of immunodeficient rats, along with survival and integration of human astrocytes into the host rat spinal cord. These cells migrated long distances, continued to divide as they migrated in host white matter, and ultimately differentiated into astrocytes that integrated into the BSCB in injured and uninjured areas of the host spinal cord. Migrated human astrocytes increased the overall astrocyte population in the host, with no observable impairment of motor function above the lesion where glia

migrated. In addition, the presence of migrated glia below the lesion did not appear to affect the improvement of motor function facilitated by synaptic relay formation.

Chapter 2: Methods

Lesion and Transplantation Surgeries. For this study, 26 adult female athymic T-cell deficient nude rats were obtained from the Harlan Laboratories. For our lesion model, we performed C5 lateral hemisections procedures that have been described previously (Lu et al., 2012; Lu et al., 2017). Prior to surgery, we anesthetized these animals using a mixture of ketamine (25 mg/ml), xylazine (1.3 mg/ml), and acepromazine (0.25 mg/ml) under sterile conditions. At the C5 segment, we performed a dorsal laminectomy and cut through the dura mater. We retracted the dura mater and performed a lateral C5 hemisection using iridectomy scissors and microaspiration, removing a 2-mm-long block of the right hemicord. The lesion was visually verified to ensure complete transection before stitching the animal closed. Postoperative treatment included daily subcutaneous ampicillin injections (3-5 mg/kg) every day for 1-2 weeks. Amoxicillin and sulfamethoxazole was mixed into drinking water (0.5 mg/ml) until sacrifice.

Human NSCs were derived from the widely used human H9 embryonic stem cell (ESC) line by methods described previously (Lu et al., 2017; Zhang et al., 2001; Thomson et al., 1998). We purchased our ESC-derived H9 human NSCs from Invitrogen and cultured them on a CELLstart coated flask from Thermo Fischer Scientific. To visualize the survival, differentiation, migration, and process outgrowth of transplanted NSCs *in vivo*, we transduced the NSCs with lentiviruses expressing the GFP reporter gene prior to transplantation, which allowed approximately 95% of NSCs to express GFP. A fibrin matrix was used consisting of fibrinogen (F6755; Sigma; 25 mg/ml) and thrombin (T5772; Sigma; 25 U/ml) to facilitate retention of NSCs in the

lesion site. In order to promote survival of the transplanted NSCs *in vivo*, the following growth factors were included in the growth factor cocktail: brain-derived neurotrophic factor (452-02; Peprotech; 50 µg/ml), neurotrophin-3 (450-03; Peprotech; 50 µg/ml), platelet-derived growth factor (P30736; Sigma; 10 µg/ml), insulin-like growth factor 1 (I8779; Sigma; 10 µg/ml), epidermal growth factor (10 µg/ml), basic fibroblast growth factor (F0291; Sigma; 10 µg/ml), acidic fibroblast growth factor (F5542; Sigma; 10 µg/ml), glial cell line-derived neurotrophic factor (G1401; Sigma; 10 µg/ml), hepatocyte growth factor (HGF; Sigma; 10 µg/ml), and calpain inhibitor (M6690; Sigma; 50 µM) (Lu et al., 2012). NSCs were harvested and mixed with the aforementioned fibrin matrix and growth factors, then injected into the lesion cavity 2 weeks after injury, which is a clinically relevant time-point.

Three animals were excluded due to death or severe illness following C5 hemisection. The remaining twenty-three subjects were randomly divided into two groups: one group receiving a human NSC graft (n=18) and one group serving as control (n=5). Animals in the experimental group were perfused either 1 month (n=3), 3 months (n=5), 6 months (n=5), 12 months (n=5) or 18 months (n=5) after grafting. Animals in the control group received C5 hemisections and injection of the fibrin matrix containing the growth factor cocktail, but without NSCs. Animals in the control group were perfused 12 months after grafting.

Histology. At varying time-points, animals were transcardially perfused with 4% paraformaldehyde in a 0.1 M solution of phosphate buffer at pH 7.4 and post-fixed overnight in the same solution at 4°C. Brains and spinal cords were dissected out and suspended in sucrose for three days. We cut out a 12 mm portion of the spinal cord with

the grafted/lesion site located in the middle for horizontal sectioning, the entire right hemisphere of the brain for sagittal sectioning, and 1-2 mm segments of C2, C8, T6, T12, and L4 of the spinal cord for coronal sectioning. All sections were cut at 30 μm thickness using a cryostat and collected serially into 24-well plates.

Immunohistochemistry. Antigen blocking and tissue permeabilization of free floating brain and spinal cord sections were accomplished using .25% Triton X-100 in Tris-Buffered Saline (TBS) for 1 hour on a shaker at room temperature, followed by incubation of primary antibodies in the same solution plus 5% donkey serum overnight at 4°C. Sections were rinsed in 3% donkey serum and .25% Triton X-100 in TBS. Alexa 488-, 594-, or 647-conjugated donkey secondary antibodies (Invitrogen, Thermo Fisher Scientific; 1:500) were incubated in the aforementioned solution for 2.5 hours on a shaker at room temperature. Sections were then washed with TBS, mounted on uncoated slides, and coverslipped with Fluoromount-G (SouthernBiotech). The following primary antibodies were used: (a) GFP rabbit polyclonal antibody (GFP-1020; Aves; 1:1000) was used to assess survival, differentiation, and migration of grafted cells; (b) hNu mouse monoclonal antibody (mab1281; Millipore; 1:500) was used to assess migration of grafted cells; (c) Ki67 rabbit polyclonal antibody (gtx16667; Genetex; 1:500) was used to assess division of migrating grafted cells; (d) Human-specific vimentin mouse monoclonal antibody (nbp2-44833; Novus Biologicals; 1:500) was used to assess differentiation of grafted cells into glial progenitor cells; (e) Human-specific glial fibrillary acidic protein (hGFAP) rabbit polyclonal antibody (TA302094; Origene; 1:500) was used to assess differentiation of grafted cells into astrocytes and involvement in the gliovascular unit (f) glial fibrillary acidic protein (GFAP) goat polyclonal antibody (sc-

6170; Santa Cruz Biotechnology; 1:500) was used to assess endogenous and migrating populations of astrocytes; (g) adenomatous polyposis coli (APC) mouse monoclonal antibody (OP80; Oncogene; 1:500) and myelin basic protein (MBP) rabbit polyclonal antibody (AB980; EMD Milliopore; 1:500) was used to assess whether oligodendrocytes migrated; (h) RECA1 mouse monoclonal antibody (ab9774; Abcam; 1:500) was used to assess the interaction of graft-derived astrocytes with host endothelial cells; (i) human-specific Nestin mouse monoclonal antibody (ab18102; Abcam; 1:500) was used to assess whether undifferentiated grafted neural stem cells migrated; (j) Platelet-derived growth factor receptor β (PDGFRβ) rabbit polyclonal antibody (sc-432; Santa Cruz Biotechnology; 1:500) was used to assess whether human astrocytes interacted with endogenous rat pericytes; (k) Connexin 43 (CX43) rabbit polyclonal antibody (48-3000; ThermoFisher Scientific; 1:200) was used to assess whether human astrocytes form perivascular astrocytic networks.

Quantification. HuNu/GFP colocalizing puncta were counted on images taken by a Keyence BZ-X710 all-in-one microscope and camera (magnification: 20x) to quantify the number of migrating human NSCs in one coronal C2 spinal cord section per subject, then averaged with other subjects in the same group to determine the mean number of cells migrated three segments rostral to the grafted/lesion site ± SEM. Ki67/GFP colocalizing puncta were counted on images taken by an Olympus Fluoview FV1000 confocal microscope (magnification: 140x) at measured distances 0-2 mm, 2-4 mm, and 4 mm + from the leading edge of cell migration for multiple sagittal brainstem and spinal cord sections per subject, then averaged with other subjects in the same group to determine the mean number of dividing cells ± SEM.

Images of the left (ungrafted) lateral region and right (grafted) lateral region in each C2 coronal spinal cord section was taken using an Olympus Fluoview FV1000 confocal microscope at (magnification: 20x) in order to quantify the amount of migrating grafted human astrocytes three segments rostral to the grafted/lesion site. GFAP expression was measured using the ImageJ software on images of fixed box size at 1600x1200 pixels. Each pixel value representing GFAP expression was converted to mm^2. The area of GFAP expression on the right (grafted) lateral region of each C2 coronal spinal cord section was divided by the reference area of GFAP expression on the left (ungrafted) lateral region on the same C2 section for each subject. The ratio of GFAP expression on the grafted side to the ungrafted side was averaged with the ratio of other subjects in the same group (6, 12, or 18 months after grafting) to determine the mean ratio per group ± SEM.

Statistics. Comparisons among groups were tested by Student's t test or non-parametric test as appropriate using the JMP statistical software. All data are presented as the mean ± SEM.

Study approval. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Veterans Administration (VA) San Diego Health Care System. National Institutes of Health (NIH) guidelines for laboratory animal care and safety were strictly followed.

Chapter 3: Results and Figures

We performed a long-term, 18-month study focused on glial maturation, migration, integration, and safety. Twenty-six T-cell deficient nude rats were used to avoid immune rejection of the human NSC xenograft. We performed C5 lateral hemisections on all twenty-six rats; three animals died due to complications after surgery. H9 human NSCs were transduced with lentiviruses expressing GFP under the ubiquitin promoter to allow visualization and tracking of NSCs after transplantation. Two weeks after injury, these human NSCs were mixed with a growth factor cocktail (see Chapter 2: Methods) to promote NSC survival and embedded into a fibrin matrix to create a gel that facilitated retention of the graft in the C5 lateral hemisection site. Eighteen rats received human NSC grafts, while five rats received a vehicle injection. The eighteen rats in the experimental group were sacrificed either 1 month (n=3), 3 months (n=3), 6 months (n=5), 12 months (n=3) or 18 months (n=4) after grafting and were compared to lesion controls. The rats in the control group (n = 5) received the C5 lateral hemisections as well as injections of the fibrin matrix containing growth factor cocktail without NSCs; these rats were sacrificed after 12 months.

Grafted human cells migrate long distances in the rostrocaudal directions. We observed gradual migration of GFP-labeled cell bodies from the lesion site over a period of 18 months. To confirm that cells were indeed migrating from the graft and not axons extending from grafted neurons, we used the hNu antibody to specifically label human nuclei (Figure 1, A-B). At 1 month after grafting, we saw few migrated cells; however, we did observe GFP-labeled axons extending 4 mm from the graft (Figure 1, C). As early as 3 months after grafting, migrated hNu/GFP double-labeled cells can be

visualized 4 mm from the graft (Figure 1, D). The number of migrated hNu/GFP double-labeled cells increased over time, and a substantial number of cells appear to have migrated 4 mm from the graft at 12 and 18 month time points (Figure 1, E-G). By 18 months, hNu/GFP double-labeled cells migrated up to 45 mm in total distance. In the caudal direction, cells migrated to the mid-thoracic level (30 mm), whereas in the rostral direction, cells migrated to the brainstem (15 mm). These cells migrated slowly, at a rate of 2-3 mm per month.

Grafted human cells migrate ipsilateral and contralateral to the hemisection site in host white matter. We used the hNu antibody to specifically label human nuclei and visualize the spatial distribution of migrated cells in coronal spinal cord sections at C2, three segments rostral to the lesion site. Interestingly, we observed migrated cells exclusively in the host white matter over the 18 month study (Figure 2, A, C, E), which is consistent with studies showing that astrocyte progenitors migrate along axonal tracts during mammalian neural development (Klämbt, 2009). After 6 months, we saw mostly graft-derived processes and very few hNu/GFP double-labeled cells at C2 (Figure 2, A-B). By 12 and 18 months after grafting, we detected cells migrating ipsilateral and contralateral to the right hemisection lesion site at C2 (Figure 2, C-F). We quantified the total number of hNu/GFP double-labeled cells in coronal sections at C2 in subjects sacrificed 6 months, 12 months, or 18 months after grafting (Figure 2G). The mean number of hNu/GFP double-labeled cells detected three segments rostral to the graft/lesion site increased over time ($P < .05$ by nonparametric comparison test).

Migrating grafted cells continue to divide at the leading edge of migration. Because glia are known to divide as they migrate during neural development (Klämbt,

2009), we used the cell proliferation marker, Ki67, to ask whether human cells grafted into the injured rat spinal cord divided as they migrated. We found that the majority of Ki67/GFP double-labeled cells were found within 2 mm from the leading edge of migration at 6 month, 12 month, and 18 month time-points (Figure 3, A-I). We quantified the number of Ki67/GFP double-labeled cells in subjects sacrificed 18 months after grafting and found an average of 3.75 $\pm$ 0.75 dividing cells within a distance of 0 - 2 mm from the leading edge of cell migration, compared to 0.25 $\pm$ 0.25 dividing cells within a distance of 2-4 mm from the leading edge, and 0 $\pm$ 0 dividing cells at a distance greater than 4mm from the leading edge ($P < 0.05$ by nonparametric test; $P < 0.05$ comparing 0-2 mm to 2-4 and >4mm, respectively) (Figure 3, J).

Migrating grafted cells differentiate into glial progenitors and astrocytes. To characterize the cell types migrating from the graft, we performed immunodetection experiments for a variety of neural cell types. We previously reported that NSCs that adopt a neuronal fate do not migrate (Lu et al., 2017). We endeavored to more fully characterize the cells migrating from the graft by using a human-specific vimentin (Vim) antibody to specifically label graft-derived glial progenitor cells and a human-specific GFAP (hGFAP) antibody to label graft-derived astrocytes in coronal C2 sections. Three segments rostral to the lesion site, Vim/GFP and hGFAP/GFP colocalization was detected by 12 months but not 6 months (Figure 4, A-B). We also used the human-specific NSC marker, Nestin, and saw very weak levels of immunoreactivity among migrating cells (data not shown). These results suggest that the vast majority of migrating cells from the lesion site adopt an astrocytic fate and begin migrating after differentiating from the neural stem cell stage to the glial progenitor stage. We stained

for the mature oligodendrocyte marker, APC (Figure 4C), and did not see any double-labeling of APC and GFP either rostral or caudal to the lesion/graft site. We also stained for the myelin marker, myelin basic protein (MBP) and did not see any double-labeling of GFP and MBP three segments rostral to the graft (data not shown). These results suggest that glia that adopt an oligodendrocyte fate remain in the lesion/graft site and do not migrate.

Graft-derived human astrocytes join the endogenous population of astrocytes. Based on a previous study of NSC transplantation in the spinal cord (Chen et al., 2015), we asked whether migrated graft-derived human astrocytes replaced the endogenous population of astrocytes in the host. To answer this question, we compared relative GFAP expression levels between the grafted and ungrafted lateral white matter regions of coronal C2 sections in each subject. Ungrafted lateral regions used as a control did not have any GFP or hGFAP expression. We observed that the relative GFAP expression on the grafted side appears to increase approximately 2-fold compared to that of the ungrafted side in subjects sacrificed 18 months after grafting (Figure 5, A-C, E). Quantification of the relative levels of GFAP expression on the grafted side relative to the ungrafted side showed a gradual proportionate increase over the time period from 6 to 18 months (Figure 5, D). In subjects sacrificed 18 months after grafting, we detected a statistically significant difference between the GFAP expression levels in the grafted side versus the ungrafted side ($P < .05$, Student's t test) (Figure 5, D). These data suggest that migrated human astrocytes join the endogenous population of astrocytes in the host rat, increasing the overall population of astrocytes where graft-derived human astrocytes were found to migrate. The increase in total astrocyte density

in our study differs from reports of human astrocytes functionally replacing endogenous mouse astrocytes when human-derived neural progenitors cells were transplanted into uninjured spinal cords (Chen et al., 2015).

Graft-derived human astrocytes form a blood-spinal cord barrier. Astrocytes, pericytes, and endothelial cells are known constituents of the BBB and BSCB, with pericytes found partially between endothelial cells and astrocytic cytoplasmic processes, also known as astrocytic perivascular endfeet (Abbott et al., 2006; Armulik et al., 2010; Bartanusz et al., 2011). Because pericytes regulate the structural integrity of the BBB and BSCB (Armulik et al., 2010), we asked whether grafted human astrocytes interact with host pericytes in forming a BSCB at the site of injury. Using the pericyte expression marker, PDGFRβ, and the human specific astrocytic marker, hGFAP, we saw human graft-derived astrocytic endfeet interacting with endogenous pericytes (Figure 6, A-C). In another experiment to determine whether human astrocytes reform a BSCB at the lesion/graft site, we stained sagittal spinal cord sections for the endothelial cell marker, RECA1, and human-specific astrocytic marker, hGFAP. We observed human graft-derived astrocyte endfeet interacting with host endothelial cells at the lesion/graft site (Figure 6, D-G). These data suggest that these graft-derived human astrocytes integrate into the host spinal cord and form a BSCB at the site of injury.

We also stained coronal C2 sections for RECA1 (for blood vessels) and hGFAP, which is three spinal cord segments rostral to the lesion/graft site where astrocytes were found to migrate. We found evidence of astrocytes wrapping their endfeet around host endothelium 18 months after grafting (Figure 6, H-I) suggesting that migrated astrocytes also contribute to the maintenance of the blood-brain barrier in regions of the CNS

unaffected by injury. To determine whether grafted human astrocytes form perivascular astrocytic networks, we stained for connexin 43 (CX43), the main gap junction protein expressed in astrocytic perivascular endfeet, which has been shown to be critical for BBB and BSCB integrity (Chen et al, 2015; Ezan et al., 2012). We detected CX43 expression among the astrocytic perivascular endfeet of migrated human astrocytes (Figure 6, J-L), suggesting the formation of functional perivascular astrocytic networks and possible involvement in intracellular trafficking of ions and signaling molecules.

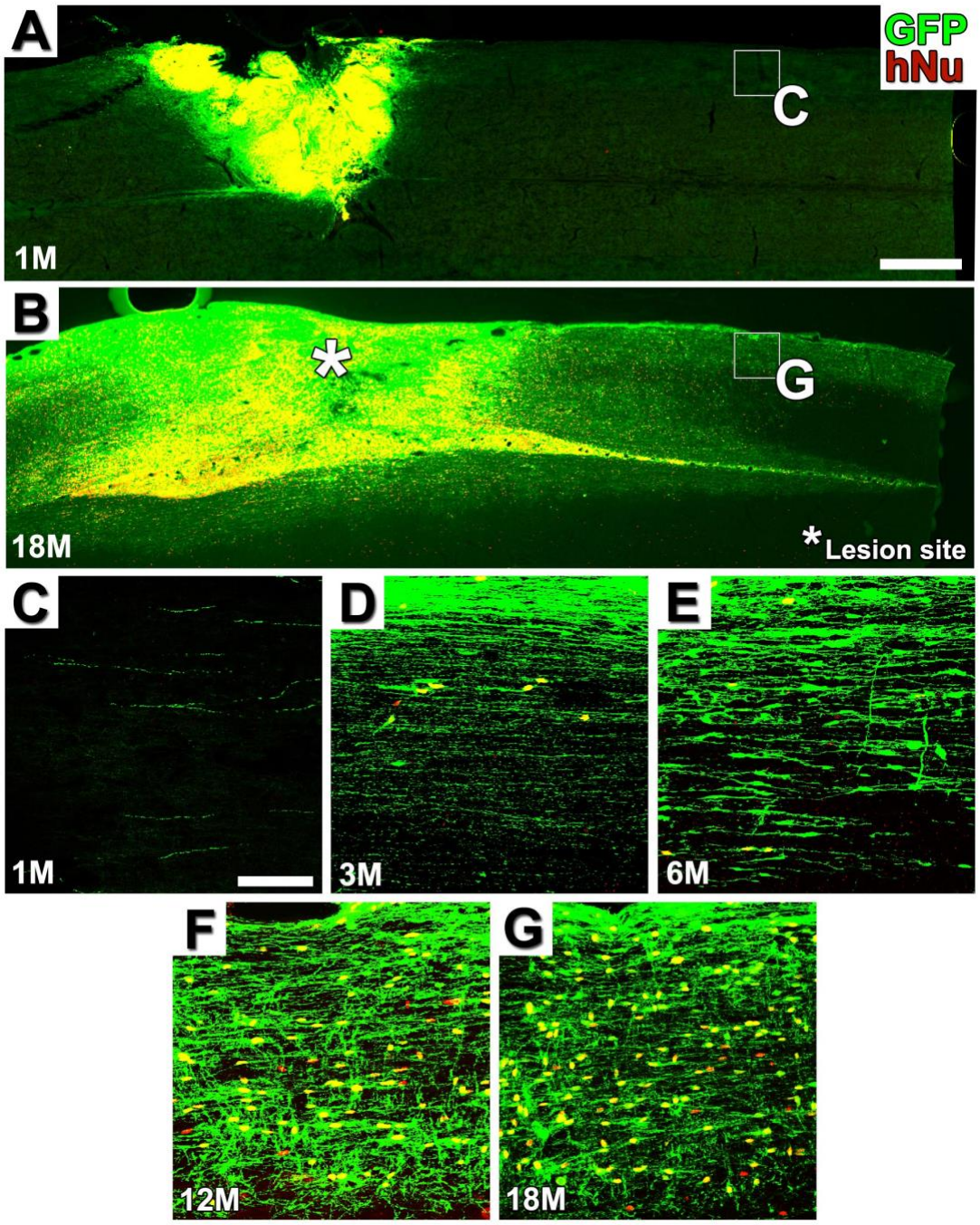

Figure 1. Grafted human neural stem cells gradually migrate from the lesion site
(A-G) hNu and GFP colabeling (horizontal sections). (A-B) Human NSCs migrate long distances from lesion/graft site through host white matter from 1 month to 18 months after grafting. (B) Asterisk represents lesion/graft site. (C-G) Higher magnification of migrating cells colabeled by hNu and GFP (C) GFP-labeled axons, but not cells, extend 4 mm caudally from the graft/lesion site 1 month after grafting. (D-G) hNu- and GFP-labeled cells gradually increase in number 4 mm rostral from the graft/lesion site 3 to 18 months after grafting. Scale bar, 1 mm (A and B); 60 μm (C-G).

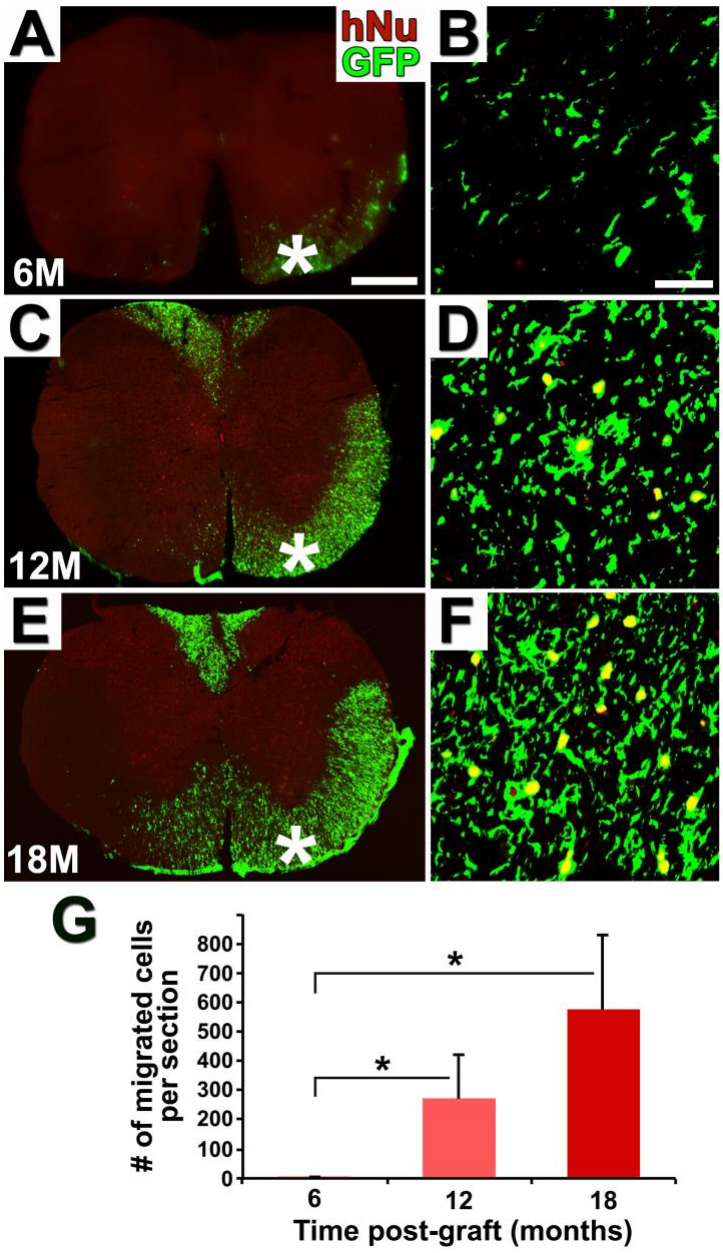

Figure 2. Spatial distribution of migrating human neural stem cells over time
(A, C, E) Spatial distribution of hNu and GFP colabeled cells in C2 coronal spinal cord sections for subjects sacrificed 6, 12, and 18 months after grafting. Asterisks represent area magnified in adjacent panels. Images were taken 3 segments rostral to the lesion/graft site. (B, D, F) Higher magnification images of coronal C2 sections for subjects 6, 12, and 18 months after grafting. (B) GFP-labeled axons, but not cell bodies, are visible 6 months after grafting. (D, F) hNu and GFP double labeled cells can be detected 12 months after grafting, and increase in number by 18 months. (G) Quantification of hNu and GFP colabeled cells per coronal C2 section. The number of migrated cells increases over a period of 18 months. These data represent the mean ± SEM. $P < .05$, by nonparametric test. Scale bar, 400 μm (A, C, E); 30 μm (B, D, F).

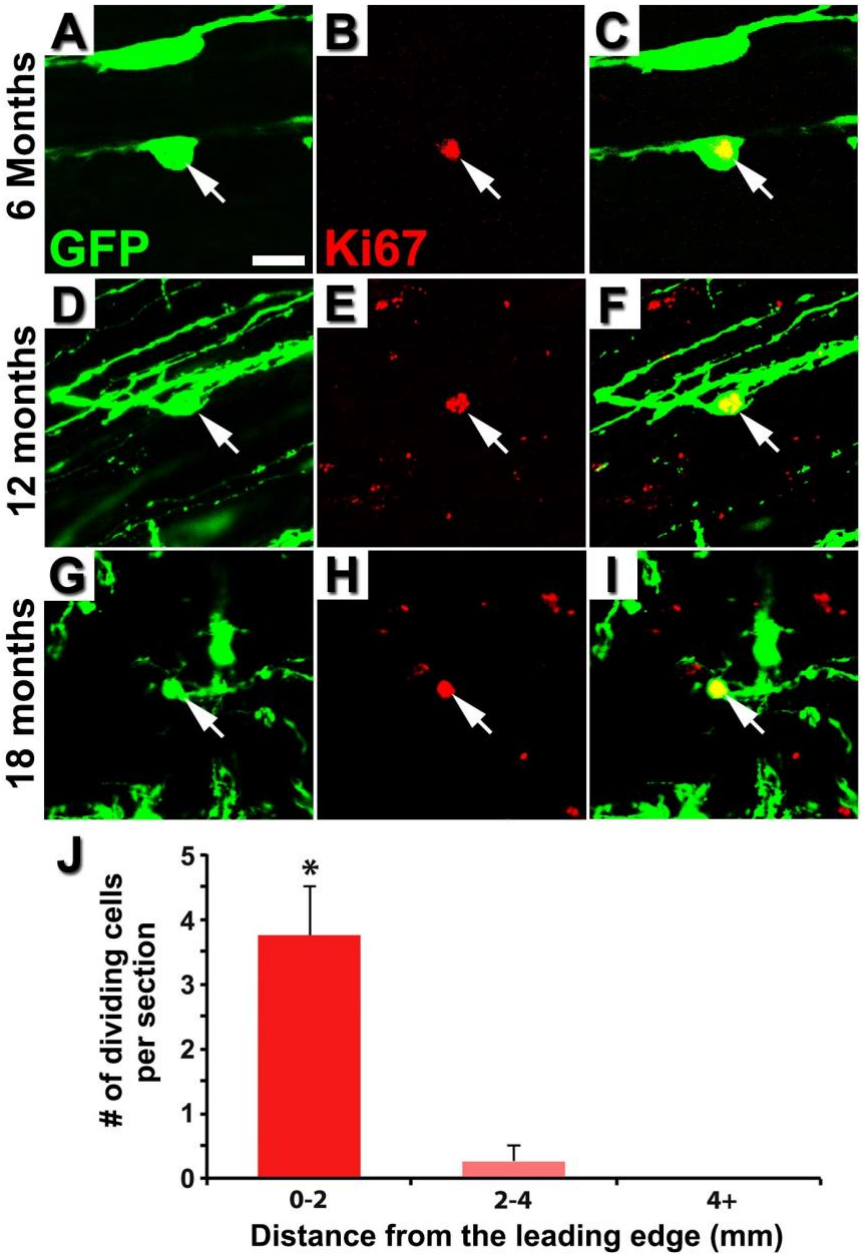

Figure 3. Human neural stem cells divide at the leading edge of migration
(A-I) Examples of Ki67 and GFP colabeled cells detected at the leading edge of migration in subjects sacrificed 6, 12, and 18 months after grafting. (J) Quantification of ki67 and GFP colabeled cells per section at the leading edge of migration 18 months after grafting. No colabeled cells were detected from 4 mm from the leading edge to the graft. These data represent the mean ± SEM. $P < .05$, by nonparametric test. Scale bar, 20 μm (A-I).

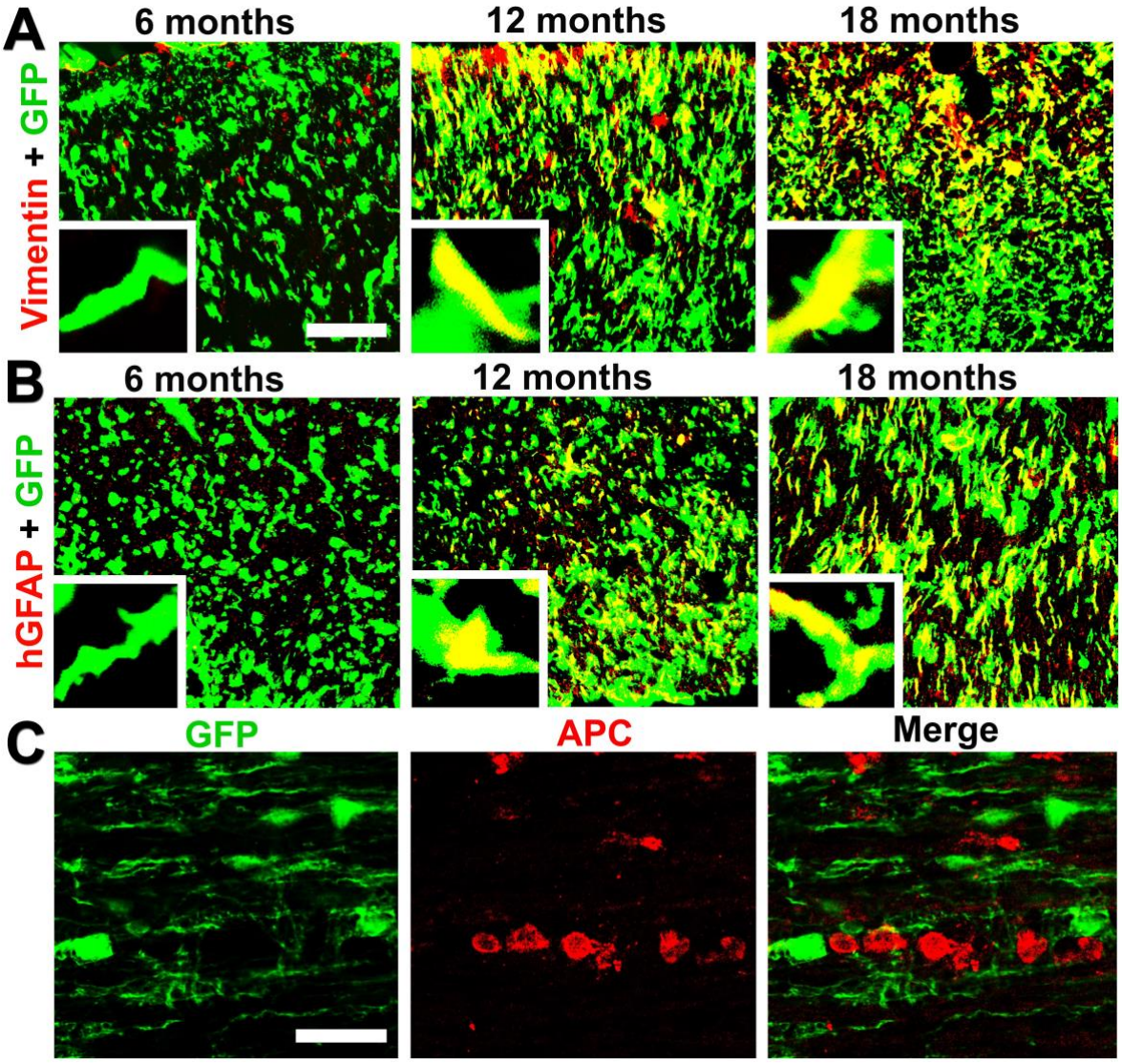

Figure 4. Migrated human neural stem cells differentiate into glial progenitors and astrocytes but not oligodendrocytes

(A-B) Coronal spinal cord sections at 6, 12, and 18 months after grafting. Images are taken 3 segments rostral to the lesion/graft site. Inset represents high magnification image of stained human glial processes. (A) By 12 months, expression of the human-specific glial progenitor marker, vimentin, appears among migrated graft-derived human cells. (B) By 12 months, expression of the human-specific astrocytic marker, hGFAP, appears among migrated graft-derived human cells. (C) The mature oligodendrocytic marker, APC, did not colabel with GFP-positive migrated graft-derived human cells. Scale bar, 30 μm (A-B); (C).

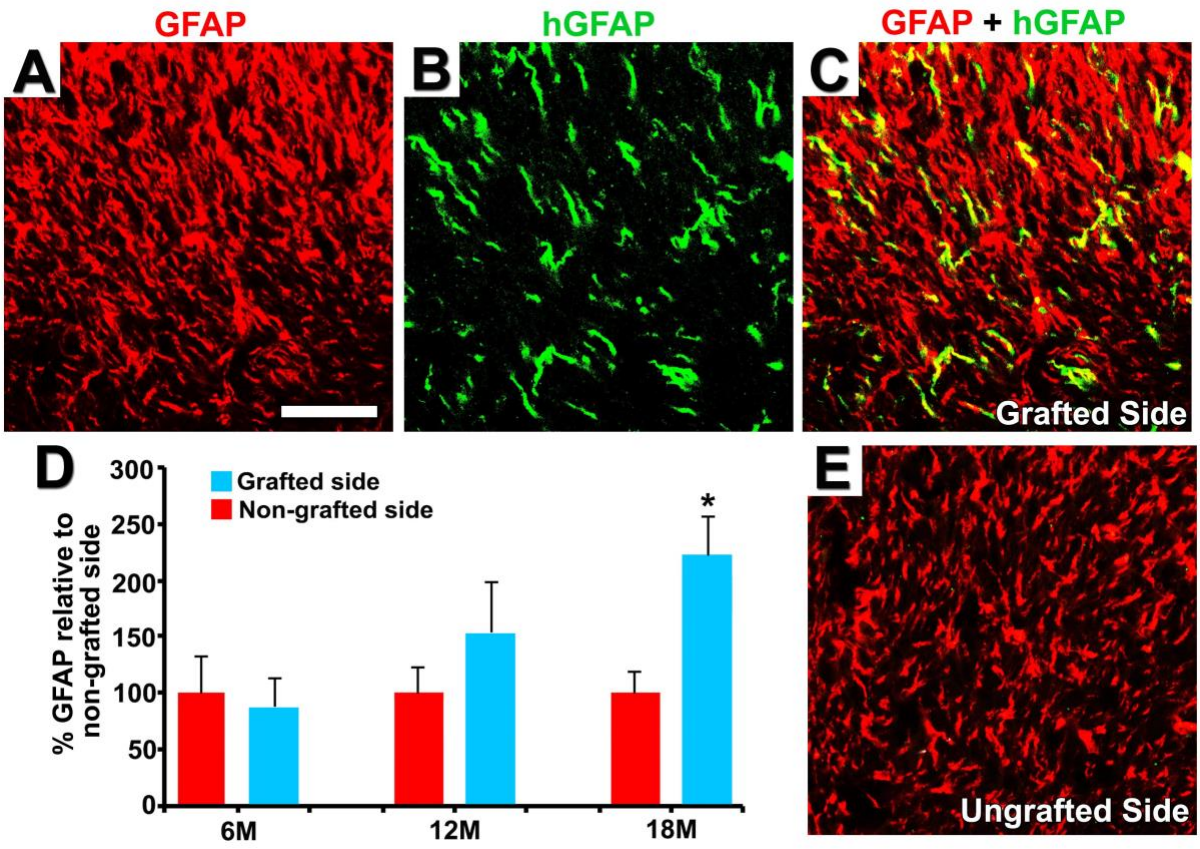

Figure 5. Migrated graft-derived astrocytes join the endogenous population of astrocytes and increase the overall glial population

(A-C, E) Comparison of astrocyte density three segments rostral to the lesion site on coronal spinal cord sections. (A) GFAP immunoreactivity on grafted side. (B) hGFAP immunoreactivity on grafted side. (C) Merging of GFAP and hGFAP immunoreactivity on grafted side. (D) Quantification of GFAP immunoreactivity between grafted side and ungrafted side. These data represent the mean ± SEM. $P < .05$, Student's t test. (E) Expression of GFAP on ungrafted side. No hGFAP expression was observed on the ungrafted side. Scale bar, 30 μm (A-C, D).

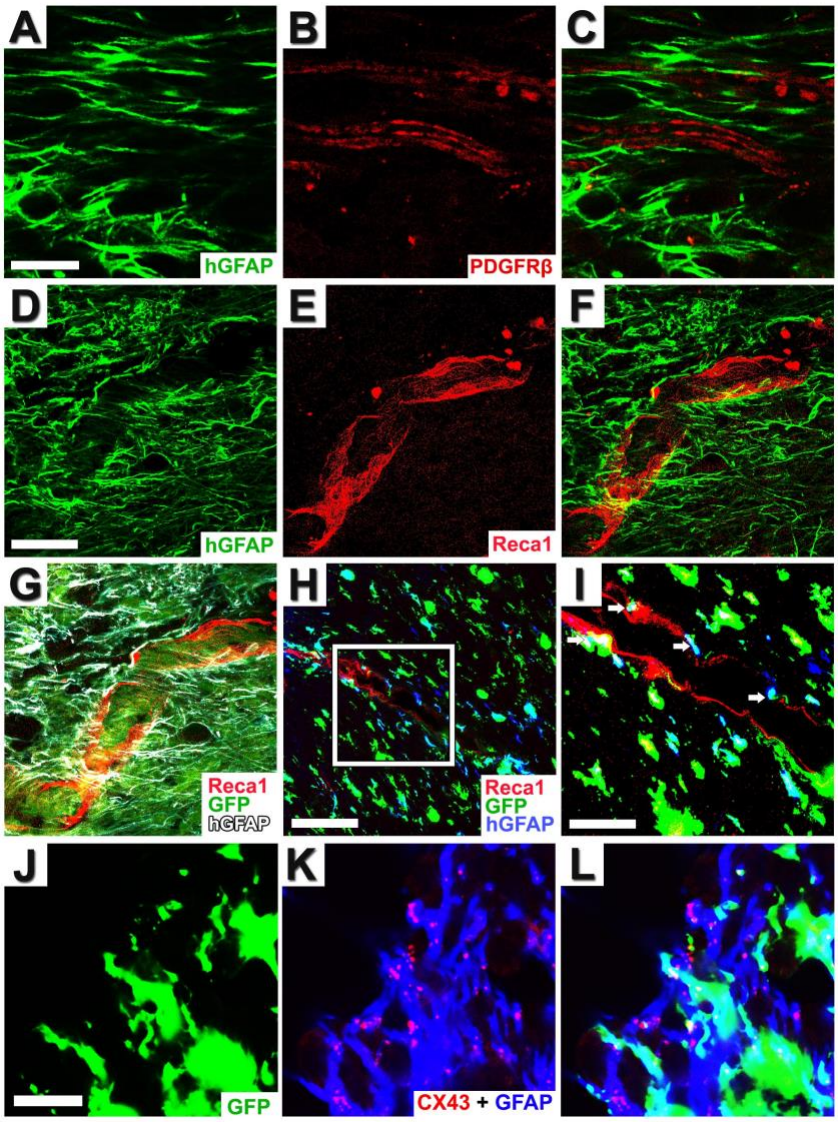

Figure 6. Graft-derived astrocytes adopt blood-spinal cord barrier phenotypes
(A-C) Astrocytic processes labeled with hGFAP interacting closely with endogenous pericytes labeled with PDGFRβ. (D-F) Astrocytic processes labeled with hGFAP ensheathe host endothelial cells labeled with RECA1 in the lesion/graft site. (G) Merged image showing multiple hGFAP labeled astrocytic processes (gray) wrapped around host endothelial cells (red) in the lesion/graft site among other GFP-labeled grafted neural stem cells (green). (H-I) Gliovascular units (in cyan) form between hGFAP/GFP colabeled processes and RECA1 labeled host endothelium 3 segments rostral to the lesion/graft site. (J-K) GFP labels all grafted human cells and processes. Grafted human and endogenous rat astrocytic processes labeled with GFAP are connected via the major astrocytic gap junction protein, CX43. (L) Merged image of human astrocytic processes labeled with hGFAP (cyan) connected to each other and to endogenous rat astrocytic processes (blue) labeled with GFAP via the astrocytic gap junction proteins labeled with CX43 (A-L). All images were taken 18 months after grafting. Scale bar, 20 μm (A-C); 50 μm (D-H); 17 μm (I); 20 μm (J-L).

Chapter 4: Discussion

After transplantation of human NSCs into rat C5 hemisection sites two weeks after injury, we observed gradual, long-distance migration and differentiation of glial progenitors and astrocytes over a period of 18 months exclusively in the host white matter. No impairment of motor function was observed above the lesion where grafted human astrocytes were found, suggesting that there were no adverse consequences of this extensive glial migration. Grafted human astrocytes extended perivascular endfeet that surrounded host blood vessels and pericytes in injured and uninjured areas of the host spinal cord. In addition, grafted human astrocytes were found to be connected via gap junctions with each other and with endogenous rat astrocytes, possibly forming functional perivascular astrocytic networks. The integration of grafted human astrocytes into the host BSCB at the injury site suggests a possible therapeutic benefit for the transplantation of glia or specifically astrocytes for addressing disruptions in the BSCB after SCI.

Migration of glia derived from human neural stem cells. The migration of transplanted human cells in our preclinical study raises important questions for future clinical trials, such as whether these cells would migrate into uninjured areas of the spinal cord in SCI patients. If transplanted cells do migrate beyond the lesion site in patients, will this compromise safety? In our study, we did not detect any adverse consequences of this extended rostrocaudal glial migration. Rostral to the lesion, there were no noticeable deficits in motor function. This is notable given that grafted human astrocytes, that migrated rostral to the lesion site, were found to surround host endothelial cells and pericytes in uninjured areas of the spinal cord, as well as form gap

junction networks, suggesting integration and participation in the host BSCB. Caudal to the lesion, migrated glia did not appear to affect late improvement of motor function; these behavioral data have been published previously (Lu et al., 2017). Moreover, Zhang and colleagues have also reported that locomotor function in mice is unaffected by migration and integration of human neural progenitor-derived astrocytes when transplanted into intact spinal cords (Chen et al., 2015). Thus, for the purposes of clinical translation, the results of our study suggest that migration and integration of graft-derived glia into the host does not raise any short-term or long-term safety concerns.

Human NSCs transplanted into non-human primates differentiate into neurons and glia; however, no glial migration was observed even by 9 months after grafting (Rosenzweig et al., 2018). Thus, astrocyte migration may be a function of species differences, in which astrocytes taken from primates and grafted into primates undergo some form of cell-mediated contact inhibition, whereas astrocytes taken from primates and grafted into rodents may migrate because rodent astrocytes are not recognized by primate astrocytes due to lack of cell-mediated contact inhibition. This is plausible due to possible evolutionary conservation of glial migratory cues in primates. In addition, migrating glia have been observed to be repelled by each other by mechanism of cell-mediated contact inhibition during neural development to ensure their even distribution throughout the CNS (Klämbt, 2009; Zhan et al, 2016). In both vertebrates and invertebrates, a common feature of glia is their ability to migrate, which can occur via cell-cell contact or cell-matrix contact (Sasse and Klämbt, 2016; Jacobsen and Miller, 2003). Therefore, another possibility for the lack of migration of primate astrocytes

grafted into primates might be explained by the presence of diffusible chemorepulsive cues. Because grafted human primate glia do not migrate in the non-human primates (Rosenzweig et al., 2018), but do migrate in rodents (Lu et al, 2017; Chen et al., 2015), we speculate that in a clinical scenario, grafted human glia would be unlikely to migrate in the adult spinal cord of a human primate. Nevertheless, the results of our study suggest that migration of graft-derived glia in humans would not be a safety concern given that none of our animals demonstrated functional deficits.

Repair of the blood-spinal cord barrier. Prolonged disruption of the BSCB is a concern after SCI due to secondary mechanisms that cause further damage to portions of the spinal cord that were not originally affected by the primary injury (Matsushita et al., 2015; Bartanusz et al., 2011; Sharma, 2005). Our findings that graft-derived astrocytes reform a BSCB at the site of injury for an extended period of time – up to 18 months after grafting – highlight an important potential benefit of NSC transplantation beyond the formation of neuronal relays for SCI. It is well documented that populations of glial progenitors exist throughout the adult mammalian CNS to maintain populations of astrocytes and oligodendrocytes over time (Goldman, 2003; Barnabé-Heider et al., 2010). The BSCB, which consists of astrocytes, has the ability to repair itself after SCI (Faulkner et al., 2004), albeit slowly on the timescale of weeks to months after injury in mammals (Matsushita et al., 2015). In animal models of SCI, the extended disruption of the BSCB leads to irreversible demyelination and loss of neural circuits (Matsushita et al., 2015; Maikos and Shreiber, 2007; Sharma, 2005). Moreover, mice lacking Aquaporin-4, a water channel widely expressed in astrocyte endfeet, weakens the BSCB and impairs motor function recovery after SCI (Kimura et al., 2010), suggesting

that the astrocyte component of the BSCB has a neuroprotective role after injury. Indeed, reactive astrocytes have been shown to be necessary after SCI to prevent weakening of the BSCB, secondary loss of neurons and oligodendrocytes, and exacerbation of motor function (Faulkner et al., 2004). These studies suggest that transplantation of astrocyte progenitors at a clinically relevant time point, such as 2 weeks post-injury, may accelerate the repair of the BSCB through replacement of damaged astrocytes. In our study, human NSCs took 6 months to differentiate into mature astrocytes, which is likely far too long to provide practical, short-term benefit in restoring BSCB structure and function. For future studies, astrocyte transplantation might be a viable therapeutic strategy for quickly repairing the BSCB after SCI and minimizing the effect of secondary degeneration of the spinal cord.

Chapter 5: Conclusion

Many preclinical studies have demonstrated the safety and efficacy of NSC transplantation for restoring motor function in rodent and primate models of SCI (Lu, 2017; Lu et al, 2012; Lu et al, 2014; Kadoya et al, 2016; Lu et al, 2017; Rosenzweig et al, 2018), highlighting the therapeutic potential of this cell replacement model. Our results show that transplanted H9 human NSCs in sites of SCI can differentiate into astrocytes that survive, migrate, and safely integrate into the host CNS. These findings suggest that astrocyte transplantation should be considered as part of a combinatorial approach for treatment of SCI, especially as a strategy to prevent secondary injury to the spinal cord due to BSCB disruption after neurotrauma. In summary, neuronal and glial replacement therapies are likely to be safe, effective, and viable ways to not only treat SCI, but other disorders of the CNS as well.

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