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Generating High Purity Embryonic Stem Cell-derived Cell Populations for Transplantation Following Spinal Cord Injury

Dylan A. McCreedy
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Generating High Purity Embryonic Stem Cell-derived Cell Populations for Transplantation Following Spinal Cord Injury

by

Dylan Alexander McCreedy

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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Dylan A. McCreedy

Washington University in St. Louis
August 2013
Dedicated to my parents. John and Diane
ABSTRACT OF THE DISSERTATION

Generating High Purity Embryonic Stem Cell-derived Cell Populations for Transplantation Following Spinal Cord Injury

by

Dylan Alexander McCreedy

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2013

Professor Shelly E. Sakiyama-Elbert, Chair

Embryonic stem cells hold great potential for cell replacement strategies in the central nervous system. Pre-differentiation into various neural cell types can help generate tissue-specific cell populations that can replace cells and tissue lost due to injury or disease. A small number of undifferentiated pluripotent stem cells persist in most transplant populations even after pre-differentiation. Given the right environment, i.e. biomaterial scaffolds, these cells can lead to tumor formation thereby eliminating any potential therapeutic benefit. This dissertation focused on the development of high purity embryonic stem cell-derived cell populations devoid of pluripotent stem cells for transplantation into the central nervous system, in particular the injured spinal cord. In the first study, transgenic expression of the puromycin resistance enzyme, puromycin N-acetyltransferase, is driven by the gene regulatory elements of the progenitor motor neuron associated transcription factor Olig2. Selection by puromycin exposure resulted in an enriched population of progenitor motor neurons, as well as recent progeny of progenitor motor neurons. Furthermore, undifferentiated stem cells were removed by puromycin selection. The efficacy of these
enriched populations was evaluated in tissue engineered fibrin scaffolds containing a heparin-based delivery system for controlled delivery of two growth factor combinations. Greater differentiation into oligodendrocytes \textit{in vitro} was observed in selected cell groups compared to unselected controls in fibrin scaffolds delivery neurotrophin-3 and glial derived neurotrophic factor. Enriched progenitor motor neurons survived and differentiated into oligodendrocytes, astrocytes and motoneurons in a two week sub-acute dorsal hemisection model of spinal cord injury. Encapsulating the transplant population in the tissue engineered fibrin scaffold with growth factors did not enhance proliferation or survival suggesting that tumorgenic cell populations were not present. In the final study, high purity mature cholinergic motoneurons were generated by driving puromycin resistance under control of two highly conserved enhancers for the motoneuron transcription factor Hb9. Puromycin selection resulted in a uniform group of post-mitotic immature motoneurons. Purity was observed through maturation and no proliferating glia were observed at any time point. Selected motoneurons maintained appropriate electrophysiological characteristics. Through this work, antibiotic selection appears to be a suitable method for generating high purity ES-cell derived neural populations.
Chapter 1

Introduction*

1.1 Overview

The goal of this dissertation was the development of high-purity embryonic stem (ES) cell-derived cell populations for use in spinal cord injury (SCI). Tumorigenicity is a major concern when using pluripotent ES cells for therapeutic applications within the body. Pre-differentiation of ES cells significantly reduces the potential for tumor formation following transplantation, however, pluripotent undifferentiated ES cells can still persist. Recent developments in biomaterial scaffolds and growth factor delivery have been shown to improve the survival, differentiation, and maturation of differentiation ES cells transplanted after SCI. Unfortunately, creating a more permissive environment can also promote the survival and proliferation of the undifferentiated ES cells that persist in the transplant population. The objective of the following research was to develop high-purity ES cell-derived transplant populations devoid of pluripotent ES cells to realize the full therapeutic potential without the downfall of potential tumor formation.

The first goal of this thesis was to develop a uniform population of progenitor cells for therapeutic application after SCI. Differentiation of ES cells usually suffers from low efficiency resulting in a heterogeneous population of cells. Subsequent purification is therefore necessary to removed undesired cell types. Progenitor cell

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populations have greater survival rates following transplantation into the injured spinal cord than terminally differentiated mature cell types and may represent an ideal therapeutic population. Progenitor motor neurons (pMNs) were targeted due to the well-studied development of this cell type and the ability to differentiate into motoneurons and oligodendrocytes, two potential therapeutic cell types for repair after SCI.

Determining the survival and differentiation of high purity pMNs within fibrin scaffolds contained growth factors and a heparin binding drug delivery system (HBDS) was the second objective of this dissertation. Fibrin scaffolds containing growth factors and the HBDS have been shown to improve the differentiation of a heterogeneous population of ES neural progenitor cells (ESNPCs) in vitro and promote the survival of the same cell population in vivo (Willerth et al., 2008, Johnson et al., 2010a). Fibrin scaffolds containing growth factors, however, may have promoted over-proliferation of undesired cell types. The effect of these components on survival and differentiation of high purity pMNs was examined after a two-week culture period in vitro. High purity pMNs were then transplanted within fibrin scaffolds into a sub-acute rodent model of SCI for two weeks to assess survival and differentiation in vivo.

The third objective of this thesis focused on the development of a high purity culture of ES cell-derived motoneurons. While pMNs show greater survival, they are also multi-potent and can give rise to motoneurons and glia. Glia can quickly proliferate and over-take in vitro cultures while the number of post-mitotic motoneurons remains constant. This greatly limits the number of studies that can be conducted using ES-cell
derived motoneurons. Isolating a single cell type also has advantages in determining mechanism of recovery following transplantation into the injured cord.

The overall objective of this work is to provide a simple, inexpensive, reproducible, and effective method for high purity ES-cell derived cell populations. While the initial intended purpose is repair of SCI, these populations have potential in toxicology screening, developmental biology, stem cell technologies, and many other fields of science. The following introduction will discuss the pathophysiology of SCI and subsequent attempts to repair the injured cord using cell transplants, in particular the use of ES-cell derived populations, and bioengineered combination therapies involving cell transplantation.

1.2 Spinal cord injury

SCI is a debilitating event resulting in disrupted motor and sensory pathways. Approximately 12,400 new cases occur annually in the U.S. adding to over 250,000 persons already living with SCI (Devivo, 2012, National Spinal Cord Injury Statistical, 2013). The main cause of SCI in the U.S. is motor vehicle-associated accidents followed by falls, violence, and sports accidents. The average lifetime cost is $69,204 per year, with more severe injuries resulting in higher medical costs. SCI injury significantly reduces life expectancy, primarily through increased susceptibility to pneumonia and infection related complications, such as septicemia. The debilitating nature of SCI injury can also reduce productivity. The employment rate of persons with SCI one year following injury is 11.7% compared to 57.1% at the time of injury. Promoting recovery following SCI can reduce total lifetime costs and enhance the overall quality of life.
Scientific research dedicated to improving regeneration following SCI has made significant strides in the past decade. The main focus of this research has been directed towards understanding the cellular and molecular events that follow SCI and the development of corresponding therapeutic strategies.

Initial mechanical trauma during SCI severs axons, damages vasculature, and promotes necrotic cell death (McDonald and Sadowsky, 2002, Thuret et al., 2006). The ensuing vascular, cellular, and inflammatory events propagate cell death in a secondary phase of injury that expands the lesion site significantly. Infiltrating Schwann cells, fibroblasts, and glia progenitors along with activated microglia and astrocytes form an interwoven cellular layer between the injured and naïve tissue (Bruce et al., 2000, Guest et al., 2005, Thuret et al., 2006). Many cells within this glial scar express chondroitin sulfate proteoglycans (CSPGs) that inhibit cell migration and axon regeneration (Jones et al., 2003). In cases of severe SCI, the necrotic tissue inside of the scar forms a cystic cavity lacking any growth promoting substrates. Regeneration following SCI is severely limited, prompting many therapeutic strategies including cell replacement therapies. The catastrophic events that advance cell death and tissue destruction following SCI are reviewed below.

1.2.1 Secondary Degeneration in Spinal Cord Injury

Initial mechanical trauma leads to several mechanisms of secondary degeneration in SCI. Due to the complex nature of SCI, the causality of each individual mechanism on cell death and tissue destruction can be difficult to discern. In many cases, the events are overlapping and intertwined. Some of the main events that
lead to cell death and expansion of the injury site following traumatic insult are reviewed below.

**Vascular Mechanisms** Disruption of spinal cord blood flow is a critical factor in the severity of SCI and the resulting cell death. Vessel damage occurs instantaneously during SCI leading to drastic changes in the vascular architecture. Hemorrhages are observed in the grey matter within 15 minutes post-SCI in human and rodents (Noble and Wrathall, 1989, Tator and Koyanagi, 1997). Hemorrhaging extends into the white matter at 24 hours and 1 week post-injury. At the epicenter of the injury, this is often accompanied by necrotic tissue and cavity formation in the grey matter with swollen axons in the white matter (Noble and Wrathall, 1989). Ischemia and edema are also observed following damage to the spinal cord vasculature in SCI (Koyanagi et al., 1989, Tator and Fehlings, 1991, Tator and Koyanagi, 1997, Rowland et al., 2008). In a cervical clip compression model of SCI in adult rats, reduced spinal cord blood flow correlated with injury severity and loss of motor function (Fehlings et al., 1989). Blood vessels were shown to be completely destroyed at the lesion epicenter 2 days post-SCI in rodents (Casella et al., 2002). In this study, neuronal loss accompanied vascular destruction. Many strategies have been developed to limit swelling and ischemia following SCI (O'Carroll et al., 2008, Saadoun et al., 2008, Fassbender et al., 2011). In a recent study in rats, preservation of axons and reduced apoptosis was achieved via expression of vascular endothelial growth factor to enhance angiogenesis (Liu et al., 2010). These studies demonstrate the fragile and unstable nature of the spinal cord microvasculature and its profound effect on cell death following SCI.
Ionic and Excitotoxic Mechanisms  Ionic imbalance is another mechanism for progressive cell death following SCI. Failure of ATP-dependent Na\(^+\) and K\(^+\) ion exchange as well as persistently activated voltage gated Na\(^+\) channels can promote depolarization following SCI (Faden et al., 1987). High intracellular [Na\(^+\)] can reverse the Na\(^+\)-Ca\(^+\) exchanger leading to accumulation of Ca\(^+\) and cell death via secondary messenger cascades (Stys et al., 1992, Park et al., 2004). To this end, Na\(^+\) channel blockers have been shown to promote white matter sparing and functional recovery following SCI (Schwartz and Fehlings, 2001, Hains et al., 2004). Glutamate excitotoxicity can also play a large role in cell death following SCI. (Wrathall et al., 1996). Failure to maintain the Na\(^+\) gradient can inhibit Na\(^+\) glutamate exchange leading to glutamate accumulation (Li and Stys, 2001). Glutamate excitotoxicity is exerted through NMDA receptors in the grey matter leading influx of Ca\(^+\) ions (MacDermott et al., 1986). AMPA receptors have been implicated for white matter injury (Agrawal and Fehlings, 1997, Park et al., 2004). The careful maintenance of ion concentrations can be drastically disrupted following SCI with several downstream consequences culminating in cell death.

Immune Reaction Mechanisms  Inflammation plays a major role in the secondary phase of cell death following SCI. The initial mechanical insult initiates cytokine expression in resident spinal cord cells leading to leukocyte recruitment. Expression of interleukin-1beta (IL-1\(\beta\)) and tumor necrosis factor-alpha (TNF\(\alpha\)) is detected by 15 minutes post-injury (Pineau and Lacroix, 2007). In mice, IL-1\(\beta\) mRNA peaks at 12 hours following spinal cord contusion injury. Astrocytes and microglia are mainly
responsible for IL-1β expression whereas TNFα is secreted by neurons and oligodendrocytes as well. Peak expression for TNFα corresponds to 1 hour post-injury and again at 28 days post-injury.

Neutrophils are the first peripheral immune responders to lesion site and infiltrate within 2-3 hours after injury. The number of invading neutrophils peaks around 24 hours (Carlson et al., 1998, Fleming et al., 2006, Donnelly and Popovich, 2008). Neutrophils release proteases and reactive oxygen species leading to non-specific degradation of injured tissue as well as surrounding uninjured tissue and cells (Taoka and Okajima, 2000). In rodents, blood-derived macrophages and activated microglia enter 3-4 days following injury (Stirling and Yong, 2008). Macrophages/microglia phagocytose apoptotic neutrophils, necrotic tissue and myelin debris. Similar to neutrophils, macrophages release proteases and reactive oxygen species resulting in non-specific cell death of uninjured cells (Alexander and Popovich, 2009). T cell and B cell recruitment during adaptive immunity also impacts recovery following SCI, however, many conflicting results exists on the role of these cells in regeneration (David et al., 2012, Wu et al., 2012, Laliberte and Fehlings, 2013). Together, innate and adaptive immunity contribute to the rapid expansion of the injury site and progressive loss of function. Despite the grim outlook following SCI cast by the overlapping and seemingly endless causes of cell death, endogenous cell activation does occur following SCI. The work on these cell types is reviewed in the next section.
1.2.2 Endogenous Spinal Cord Stem Cells following SCI

Recovery of function in lower vertebrates is dependent on proliferation of progenitor cells in the central canal to repopulate the spinal cord. In zebrafish, Olig2$^+$ cells in the central canal give rise to new motoneurons following SCI (Dias et al., 2012). In mammals, proliferation of progenitor cells at the central canal increases significantly after high thoracic transaction of the dorsal finiculus (Johansson et al., 1999). Meletis and colleagues performed an elegant study to determine the source of progenitor cells from the central canal. Using conditional reporter mice expressing cre recombinase under the nestin reporter, ependymal cells lining the central canal were identified as multi-potent progenitors giving rise to oligodendrocytes and astrocytes following SCI (Meletis et al., 2008). While a small portion of these cells can repopulate lost cell populations, many remain undifferentiated or contribute to glia scar formation and expression of CSPGs. No neuronal differentiation from endogenous stem cells has been observed in rodent or non-human primate SCI (Yang et al., 2006, McDonough and Martinez-Cerdeno, 2012). Beyond stem cells, endogenous NG2$^+$ glial progenitors increase proliferation after SCI and give rise to astrocytes, oligodendrocytes, and microglia (Horner et al., 2000, Zai and Wrathall, 2005).

The increase in proliferation following injury suggests that repopulation is possible following SCI. Unfortunately, neuronal differentiation is not observed and many of the new cells contribute to scar formation. While remyelination is observed in some studies, it is unclear to what extent this occurs. As a result, cell transplantation has become a viable therapeutic to replace lost populations. In the next section, we will
review the main cell types used for replacement, and demonstrate the therapeutic value of each.

1.3 Cell Replacement Strategies

A wide variety of cell types have been examined for their therapeutic potential towards repair of the injured spinal cord. Schwann cells and olfactory ensheating glia were originally investigated due to their known potential for regeneration in nervous system injuries. Other cell types, including bone marrow stromal cells, have been studied based on the ease of deriving autologous populations thereby avoiding implant rejection. More convincing studies of late have sought to utilize cells common to the spinal cord, such as neural progenitor cells, with known functions in the central nervous system (CNS). With the exception of autologous cells, all transplants require immune suppression to prevent rejection. This commonly occurs through administration of cyclosporin A, however, the therapeutic effect of cyclosporin A alone has been previously demonstrated (McMahon et al., 2009). Carefully controlled studies are necessary to discriminate the effects of cell transplants from cyclosporin A. In this section we will review the main cell types used for SCI repair.

1.3.1 Schwann Cells

Schwann cells are the myelinating glia of the peripheral nervous system (PNS). During injury to the PNS, Schwann cells help phagocytose myelin debris and cooperate with fibroblasts to form bands of Bungner that promote regeneration of ascending and descending nerve axons (Parrinello et al., 2010). Schwann cells express a variety of
growth factors in peripheral nerve injury to promote axon regeneration including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (Heumann et al., 1987, Friedman et al., 1992, Meyer et al., 1992).

Following SCI, Schwann cells infiltrate into the lesion center and occasionally remyelinate spared axons in the peripheral white matter (Bresnahan et al., 1976, Bunge et al., 1994, Beattie et al., 1997, Bruce et al., 2000). Schwann cells can be harvested from peripheral nerves and expanded in vitro (Morrissy et al., 1991). Furthermore, autologous Schwann cells from the same SCI patient can be taken to reduce immune rejection (Bunge, 2002). In complete transaction models of SCI in rats, transplanted Schwann cells contained within polyacrylonitrile/polyvinylchloride (PAN/PVC) conduits improved regeneration of propriospinal neurons into the conduit (Xu et al., 1995). In a follow up study, regeneration was observed from both the rostral and caudal ends of the severed spinal cord (Xu et al., 1997). Significant recovery of function by Schwann cell transplantation without additional factors used in combination, however, has only been reported in a few studies (Takami et al., 2002, Barakat et al., 2005, Schaal et al., 2007). Combination therapies involving Schwann cells will be reviewed in the final section of the introduction.

Schwann cells derived from non-nerve autologous sources have shown promise for repair following SCI. Skin-derived precursors (SKP), a potential neural crest stem cell analogue, can be expanded from mammalian skin and retain the ability to differentiate into Schwann cells (Fernandes et al., 2004, McKenzie et al., 2006). When transplanted in a contusion model of SCI, SKP-derived Schwann cells increase the number of serotinergic fibers at the injury site and improve remyelination both at the
injury site and in the spared rim of white matter (Biernaskie et al., 2007). Small but significant improvements were observed similar to those seen with nerve-derived Schwann cells. Overall, Schwann cells have yet to live up to their potential for regeneration observed following peripheral nerve injury. Regardless, human clinical trials are proceeding to examine the efficacy of this cell type for clinical repair of SCI (Saberi et al., 2008). Properly conducted clinical trials may elucidate the role of autologous Schwann cell transplantation in repair of SCI.

1.3.2 Olfactory Ensheathing Glia

Olfactory ensheathing glia, also known as olfactory ensheathing cells (OECs), help peripheral olfactory axons migrate into the central nervous system olfactory bulb following transection. The ability to promote regeneration into the central nervous system is maintained through life (Doucette, 1990, 1991). Similarly, OECs have been shown to promote regeneration of axotomized dorsal root axons into the spinal cord (Ramon-Cueto and Nieto-Sampedro, 1994). Mixed OEC cultures transplanted into a cervical lateral hemisection injury appeared to induce regeneration of corticospinal tract axons by creating a cellular bridge across the lesion (Li et al., 1997). OEC myelination of axons was observed; however, behavioral outcome was not reported. Transplantation of OECs into a thoracic complete transaction injury led to improved motor function and elongation of serotonergic axons (Ramon-Cueto et al., 2000). In a later study, recovery of hindlimb function and regeneration of serotonergic axons up to the caudal stump were observed following OEG transplantation and treadmill rehabilitation after
complete thoracic transaction SCI in rats. OEG transplantation alone, however, was not able to elicit a similar response.

When compared to Schwann cells, OECs exhibited a lesser impact on recovery of function following thoracic contusion injury (Takami et al., 2002, Barakat et al., 2005). Increased fiber regeneration, however, was observed following transplantation of OECs into the transected thoracic spinal cord. Transplantation of olfactory nasal mucosa, a common source of OECs, has been shown to improve recovery of motor function following a thoracic full transection SCI (Lu et al., 2001, Lu et al., 2002). Independent verification of these results, however, failed to replicate similar recovery of locomotion (Steward et al., 2006). While OECs do appear to demonstrate therapeutic potential, it is limited and may be better suited for guiding axons between the PNS-CNS interface. The lack of easily attainable autologous sources may limit the clinical translation of OECs.

1.3.3 Bone Marrow Stromal Cells

The therapeutic potential of bone marrow stromal cells (BMSCs) for repair after SCI has been heavily debated. Much of this stems from whether or not BMSCs retain the ability for differentiation into the neural lineage. Lu and colleagues demonstrated that expression of neural markers in BMSCs in vitro was not sustained following transplantation into the injured cord (Lu et al., 2005). Furthermore, neurally induced BMSCs did not differ from naïve BMSCs 1 month post-grafting. BMSCs do, however, fill the lesion cavity and secrete growth factors that may improve regeneration indirectly. In some studies, BMSCs were shown to promote improved functional recovery (Chopp
et al., 2000, Hofstetter et al., 2002). Despite early promise, behavioral data and histology following transplantation of BMSCs into the injured cord is variable (Tetzlaff et al., 2011). The ease of obtaining autologous BMSCs has ushered their use into the clinical setting, though this may be somewhat premature (Harrop et al., 2012). BMSCs do appear to help reduce cystic cavity formation and secrete growth factors that may play a role in neuroprotection and plasticity following SCI. No further evidence on any specific neural functionality, including neural differentiation and synaptic connectivity with host neurons, has been demonstrated to date.

1.3.4 Neural Stem Cells

Replacing lost spinal cord cell populations with phenotypically similar cell types has great potential for recovery of function in SCI. Neural stem/progenitor cells (NSCs) and fetal spinal cord tissue represent two pre-clinical sources of neural cells including neurons, astrocytes, and oligodendrocytes. NSCs can be amplified from CNS tissue in the presence of fibroblast growth factor-2 and epidermal growth factor to form neurospheres (Reynolds and Weiss, 1992, Mothe et al., 2008). More specific neuronal restricted precursors (NRPs) and glial restricted precursors (GRPs) can be obtained from fetal spinal cord tissue by immunopanning for the cell surface markers PS-NCAM or A2B5 respectively (Cao et al., 2002, Cao et al., 2005).

Engraftment of fetal spinal cord tissue following SCI typically fills the spinal cord lesion and functional connections are established between host and graft neurons in both rodent and feline models of SCI (Houle and Reier, 1989, Reier et al., 1992). Recently, rat spinal cord fetal tissue has been used to demonstrate long distance axon
extension from engrafted neurons into the host tissue following complete thoracic transection (Lu et al., 2012). Fetal spinal cord tissue contains many interneurons that can relay ascending and descending signals through the injury site. Despite the often positive observations, the lack of a suitable equivalent human tissue source has hindered the development of this strategy.

NSCs can be generated from both embryonic and adult CNS tissues. In addition, different CNS regions can give rise to NSCs including the subventricular zone (SVZ) of the striatum and the periventricular zone of the spinal cord (Reynolds and Weiss, 1992, Mothe et al., 2008). Transplantation of rat NSCs from the embryonic cortex or adult subventricular zone following sub-acute contusive injury resulted in primarily astrocyte differentiation with no oligodendrocytes or neurons (Cao et al., 2001). Sub-acute engraftment of adult mouse NSCs from the SVZ into compressive spinal cord injury, however, produced mainly oligodendrocyte differentiation, enhanced remyelination, and improved motor function as assessed by the Beattie-Basso-Bresnahan (BBB) locomotor scale (Karimi-Abdolrezaee et al., 2006, Parr et al., 2008). Human NSCs from fetal brain have also been shown to promote locomotor recovery (Cummings et al., 2005). In this study, human NSCs differentiated into neurons and formed contacts with host neurons in a mouse contusive SCI model. Differentiation into oligodendrocytes and subsequent myelination was also observed. In some cases, increased sensitivity to noxious stimuli or allodynia was observed, and this may limit the efficacy of NSC-based therapies (Hofstetter et al., 2005, Macias et al., 2006). Some of the variability observed in response to NSC transplantation may be explained by age, species, and region specific differences in NSCs, along with the variability of in vitro
culture conditions of NSCs. Immortalized human NSC lines may ultimately provide a consistent source for clinical translation of NSC-based therapeutic strategies; however, these are not autologous and pose the risk of immune rejection.

NRP and GRP populations provide more restricted lineage cells for SCI repair compared to NSCs. NRPs, unfortunately, show poor survival and differentiation into neurons following transplantation into the contused spinal cord (Cao et al., 2002). Similarly, GRPs are limited by predominant differentiation into astrocytes within the injured cord (Han et al., 2004, Hill et al., 2004, Enzmann et al., 2005, Tetzlaff et al., 2011). Mixtures of NRPs and GRPs can promote recovery of function, however, the advantage of these cells over NSCs is unclear (Mitsui et al., 2005, Neuhuber et al., 2008). Furthermore, the ethical limitations of suitable human fetal tissue render NRP/GRP therapies unlikely in the near future.

1.3.5 Embryonic Stem Cell-derived Neural Populations

The idea of replacing damaged neural tissue after SCI has prompted investigation into ES cell-derived neural cells. ES cells can be expanded indefinitely and give rise to all tissues of the body. McDonald and colleagues first demonstrated the viability of neurally-induced ES cells transplanted in contusive rat SCI (McDonald et al., 1999). Cells survived and differentiated into astrocytes, oligodendrocytes, and neurons. Partial recovery of function was demonstrated by BBB and gait analysis. In a follow-up study, ES cell-derived oligodendrocytes were shown to myelinate demyelinated axons suggesting a potential mechanism for recovery (Liu et al., 2000). In addition, authors manipulated the in vitro culture conditions to create oligospheres, which are cellular
aggregates highly enriched for oligodendrocytes. ES cells induced into the neural lineage have also been shown to improve both spontaneous and evoked pain behaviors in spinal cord injured mice (Hendricks et al., 2006).

High purity human ES cell-derived oligodendrocyte progenitor cells (OPCs) have been generated for repair and remyelination. Following contusive thoracic SCI, sub-acute transplantation of OPCs lead to greater myelination of spared axons and increased motor function (Keirstead et al., 2005). Treatment of chronic SCI with OPCs had no effect on remyelination and recovery. Transplantation of human OPCs into a cervical SCI improved tissue sparing and motor function while reducing cavitation (Sharp et al., 2010). Acute transplantation of human OPCs following contusive SCI promoted recovery of sensory pathways as shown by increased somatosensory evoked potential amplitudes and reduced latencies (All et al., 2012). Murine OPCs have also been demonstrated to improve remyelination and motor function following a cervical X-ray irradiation model of SCI (Sun et al., 2013).

Recently, human ES-derived OPCs and motoneuron progenitors (MPs) were compared following transplantation into a complete thoracic SCI (Erceg et al., 2010). Both groups differentiated into oligodendrocytes, neurons, and astrocytes. In addition, both groups improved functional recovery despite poor survival (<1%). In this study, poor neuronal differentiation of MPs was observed in the spinal cord. A protocol for high purity human ES-derived MPs has been developed by the Keirstead laboratory and was shown to enhance neuronal survival and recovery of functional following cervical SCI through neurotrophic and cell signaling mechanisms (Rossi et al., 2010). Murine ES cell-derived motoneurons have been shown to survive and extend axons out into
peripheral nerve following transplantation in a rodent model of amyotrophic lateral sclerosis (ALS) (Harper et al., 2004, Deshpande et al., 2006). Similar results have yet to be achieved following SCI.

The ethical concerns over the use of human ES cells have prompted investigation into the therapeutic potential of human induced pluripotent stem cells (hiPSCs) for repair after SCI. Following neural induction of human ES cells, dissociation in the presence of media hormone mix generated ES cell-derived neurospheres (Kumagai et al., 2009). Similar steps can be taken to form hiPSC neurospheres (hiPSC-NS). Sub-acute transplantation of hiPSC-NS into the contused thoracic spinal resulted in differentiation of cells mainly into neurons and astrocytes, with a few oligodendrocytes (Nori et al., 2011). Enhanced angiogenesis and tissue sparing was observed. Grafted cells appeared to make synaptic connections with host neurons. Locomotor recovery as shown by the Basso Mouse Scale and electrophysiology was significantly improved in animals receiving hiPSC-NS transplants. In a later study by the same group, functional recovery was also shown following transplantation of hiPSC-NS into a common marmoset SCI (Kobayashi et al., 2012). Neuroepithelial-like stem (NES) cells can also be obtained from hiPSCs and give rise to multiple neural lineages (Falk et al., 2012). Sub-acute grafting of hiPSC-NES cells enhanced functional recovery following SCI in immunodeficient mice (Fujimoto et al., 2012). Neural differentiation of hiPSC-NES cells was observed and was associated with neuronal sparing. While few phenotype specific functions (i.e. myelination by oligodendrocytes) are currently observed in studies utilizing hiPSCs, the demonstrated
behavioral benefits suggest great potential for hiPSCs as a cell therapy for repair following SCI.

1.4 Embryonic Stem Cells

ES cells give rise to all somatic cell types and have demonstrated immense value as a tool for studying development and as a therapeutic agent. Martin Evans and Matthew Kaufman first cultured cells from the inner cell mass of late mouse blastocysts in media conditioned by teratocarcinoma stem cells (Evans and Kaufman, 1981). When injected subcutaneously, these cells formed teratocarcinomas containing all three germ layers thus demonstrating pluripotency. The term “embryonic stem cell” was coined by Gail Martin shortly afterward who independently demonstrated similar results (Martin, 1981). The growth of mouse ES cells required co-culture on mytomycin-C inactivated fibroblasts feeder layers with media conditioned by teratocarcinomas to maintain their pluripotent states. The use of media conditioned by buffalo rat liver cells containing differentiation inhibiting activity (DIA) eliminated the need for fibroblasts feeder layers (Smith et al., 1988). DIA was later replaced by leukemia inhibitory factor (LIF) (Williams et al., 1988).

Thomson and colleagues first derived non-human primate ES-cells from Rhesus monkeys in 1995 (Thomson et al., 1995). Primate ES-cells maintained pluripotency when cultured on irradiated mouse embryonic fibroblast feeder layers in the presence of LIF. Unlike mouse ES cells, primate ES cells fail to survive in the absence of feeder layers. Thomson later derived human ES-cells, bringing ES cell-derived therapeutics closer to the clinical setting (Thomson et al., 1998). Much controversy exists over the
use of human ES cells. Recent advances in epigenetics have demonstrated reversion of somatic cells, typically fibroblasts, back to a stem cell state via the expression of genes associated with pluripotency including Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). These aptly named induced pluripotent stem cells (iPSCs) can generate cells from all three germ layers and may provide a suitable and ethical replacement for human ES cells. Generating iPSCs from patient skin cells can allow for autologous cell transplants thereby reducing immune rejection. The following section reviews the differentiation of ES cells and iPSCs into the neural lineage including derivation of specific neuronal subtypes.

1.4.1 Neural Induction of ES Cells

Neural differentiation of ES cells can be achieved by aggregating ES cells in the absence of LIF or other differentiation inhibitors. Multi-cellular aggregates of ES cells, or embryoid bodies (EBs), mimic developmental events in the embryo and start a default neural differentiation program in ES cells (Hemmati-Brivanlou and Melton, 1997). Work by the Gottlieb laboratory has demonstrated efficient differentiation of mouse ES (mES) cells in EBs into neural progenitors through exposure to retinoic acid (RA) (Bain et al., 1995). RA induced mES cells were shown to differentiate into oligodendrocytes, astrocytes, inhibitory GABAergic interneurons, and less frequently into excitatory and cholinergic neurons (Fraichard et al., 1995, Strubing et al., 1995). Increasing RA concentration was shown to further caudalize mES cells from a hindbrain to spinal cord cell fate (Okada et al., 2004). RA concentration also influences the expression of various progenitor, neuronal, and glial markers. Neural differentiation
with RA has also been shown in human ES (hES) cells (Schuldiner et al., 2000, Schuldiner et al., 2001). Highly efficient neural induction of hES cells and hiPSCs has been shown following dual exposure of the bone morphogenic protein inhibitor, Noggin, and the activin/nodal inhibitor, SB431542 (Chambers et al., 2009). This dual SMAD inhibition protocol gives rise to neural crest and CNS progenitors, with the latter showing subsequent differentiation into motoneurons and dopaminergic neurons. Neural induction by SB431542 was demonstrated to impose a caudal positional identity in the resulting neural progenitors (Patani et al., 2009). Efficient induction into general neural progenitor phenotypes can be accomplished by exposure to developmental signals including retinoic acid or by manipulating signaling pathways common to development.

1.4.2 Embryonic Stem Cell-derived Neuronal Subtypes

Cues from developmental biology have often guided the directed differentiation of ES cells into specific neuronal subtypes. Sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF-8) have been shown to induce dopaminergic differentiation in the forebrain and midbrain (Ye et al., 1998). Reproducing Shh and FGF-8 signaling in vitro promoted differentiation of mES and hES cells into dopaminergic neurons (Lee et al., 2000, Perrier et al., 2004, Yang et al., 2008). Induction protocols for the differentiation of cortical neurons (Gaspard et al., 2009), cerebellar neurons (Salero and Hatten, 2007), dorsal interneurons (Murashov et al., 2005), retinal rod and cone neurons (Osakada et al., 2008), and peripheral neurons (Lee et al., 2007) from ES cells have been developed, though not all protocols mimic developmental events. The use of chemically defined
components in published induction protocols is critical to the clinical translation of therapeutic ES cell-derived cultures. Divergent induction protocols for the same cell type often result from the use of poorly defined reagents or factors secreted by co-cultured cells types, such as stromal cells (Erceg et al., 2009). Consistency in differentiation protocols can significantly enhance consensus between studies using ES cell-derived populations.

1.4.3 Embryonic Stem Cell-derived Motoneurons

The differentiation of ES cell-derived motoneurons has been highly consistent over the past decade. Motoneurons differentiate from a distinct population of cells in the ventral neural tube called progenitor motoneurons (pMNs) (Dessaud et al., 2008). The pMN domain forms in response to RA released from nearby somites and Shh released by the notochord and floorplate cells. While differences in the initial neural induction of ES cells exists (RA vs. dual SMAD inhibition), published induction protocols rely on Shh signaling to induce differentiation into motoneurons as originally published for mES cells by the Jessell group (Wichterle et al., 2002). Differentiation of hES cells into motoneurons was demonstrated shortly after (Li et al., 2005, Shin et al., 2005). The electrophysiological properties of mES cell-derived motoneurons mimic those observed in the spinal cord including the ability to fire multiple action potentials (Miles et al., 2004). Shh can be replaced with small molecule agonists of the Shh signaling pathway, including purmorphamine and smoothened agonist (SAG), leading to more efficient conversion of ES cells and hiPSCs into motoneurons (Li et al., 2008, Wichterle and Peljto, 2008, Hu and Zhang, 2009).
The positional identity of RA and Shh-induced motoneurons was analyzed by Peljto and colleagues and determined to be cervical spinal level motoneurons expressing predominantly Hox5a (Peljto et al., 2010). This reflects the normal developmental dependence of the hindbrain and cervical spinal cord on RA signaling. More caudal thoracic and lumbar motoneurons were generated using a RA free induction that relied on endogenous Wnt and FGF signaling for caudal specification. The absence of RA also shifted cells from a predominant medial motor column identity to a more lateral medial column identity. In a recent study, combining small molecule agonists purmorphamine and SAG during induction of hES cells and hiPSCs promoted a limb innervating motoneuron phenotype compared to induction with the recombinant Shh protein alone (Amoroso et al., 2013). In this case ES cells and hiPSCs were first neurally induced by dual SMAD inhibition. All conditions led to a cervical specification of motoneurons. Many different sub-types of motoneurons can be differentiation from mES cells, hES cells, and hiPSCs for position specific applications.

1.5 Combination Therapies for Spinal Cord Injury

Several strategies have been developed to address the individual aspects of CNS trauma including limiting inflammation and secondary injury, remodeling injured tissue, neutralizing inhibitory molecules, increasing trophic support and replacing neural cell populations. Functional recovery in studies targeting a single component, however, is often modest. Combining therapies may help overcome multiple barriers to regeneration and provide synergistic effects on functional recovery. Here we review the most common and recent combination therapies that include the use of two or more
individual strategies to promote regeneration. While the majority of the work has been performed in SCI, successful therapies can be extrapolated to other types of CNS trauma.

1.5.1 Cell Transplantation and Biomaterial Combinations

Cell transplantation and biomaterial scaffolds each have unique advantages as therapeutic strategies for SCI. Cells can provide a large repertoire of signaling molecules, including anti-inflammatory cytokines and neurotrophic factors. However, cells often fail to provide topographical guidance of regenerating axons resulting in random growth (Blesch et al., 2002). Biomaterial scaffolds can guide regenerating axons but cannot replace cell populations lost due to injury. Combining cellular and material strategies may provide synergistic effects and enhance recovery following SCI. Additionally, scaffolding can serve as a vehicle for cell transplantation, enhancing survival and engraftment at the injury site.

Many groups have demonstrated improved regeneration following transplantation of Schwann cells directly into the spinal cord, however recovery is often modest (Takami et al., 2002). Schwann cells play important roles in peripheral nerve and spinal cord injuries including debris clearance and trophic support of regenerating axons (Xu et al., 1995, Oudega and Xu, 2006). The addition of Schwann cells to biomaterial scaffolds as a combination therapy may enhance recovery following SCI. In several studies, Schwann cells were reported to promote neural regeneration through poly (lactic co-glycolic) acid (PLGA) scaffolds implanted into a complete transection model (Moore et al., 2006, Olson et al., 2009, Chen et al., 2011a), however; functional recovery
was not improved (Olson et al., 2009). One potential benefit of combining cellular transplantation and scaffolds is the improved survival of transplanted cells. The effect of scaffold composition on Schwann cell survival in the spinal cord lesion was tested following contusive spinal cord injury (Patel et al., 2010). Greater cell survival, neurofilament density within the lesion, and functional recovery were observed when Schwann cells were transplanted in Matrigel compared to no scaffold or methylcellulose (MC). Matrigel, however, is generated from a sarcoma cancer cell line and is not approved for clinical use (Kleinman et al., 1982, Kleinman et al., 1986).

Transducing Schwann cells to express neurotrophins prior to seeding within scaffolds can also be used in combination strategies. Schwann cells modified to express glial derived neurotrophic factor (GDNF) have been shown to decrease glial scarring and increase neural regeneration (Deng et al., 2011). When mixed with Matrigel and seeded into guidance channels of PAN/PVC scaffolds, GDNF-expressing Schwann cells induced migration of host astrocytes into the scaffold and reduced the presence of CSPGs at the scaffold interface. In another study, Schwann cells expressing NT-3 improved neuronal survival and locomotor recovery when injected into collagen scaffolds with NSCs. Independent verification is necessary to determine the clinical applicability of combination therapies involving neurotrophins, Schwann cells, and biomaterial scaffolds. Furthermore, the use of Schwann cells is limited by the lack of suitable sources. In most studies, Schwann cells are isolated from peripheral nerves requiring loss of function at the donor site (Kreider et al., 1981). Several weeks are needed to expand Schwann cells to obtain a sufficient number of cells for
transplantation, thus limiting their use in acute treatments. Additional work is therefore needed to generate alternative sources of Schwann cells.

Transplantation of BMSCs has been shown to reduce cavitation of the injury site, enhance regeneration, and promote functional recovery (Hofstetter et al., 2002, Tohda and Kuboyama, 2011). Recent studies have tested the efficacy of BMSCs in combination strategies utilizing scaffolds. When seeded in chitosan conduits, BMSCs led to improved spinal cord motor evoked potential amplitude (Chen et al., 2011b). However, no significant improvements were observed in locomotor recovery. Furthermore, BMSCs reduced the lesion size and the presence of macrophages. In another study, BMSCs expressing BDNF improved GAP-43+ fiber regeneration in Matrigel scaffolds and greater functional recovery versus Matrigel scaffold controls, but were not statistically different from unmodified BMSCs in Matrigel (Koda et al., 2007). Human BMSCs transplanted in collagen scaffolds reduced the lesion size and improved spatial learning and functional recovery following traumatic brain injury (TBI) (Lu et al., 2007). While BMSCs show promise for repair following SCI and TBI, inconsistencies in reported locomotor recovery currently limits their use.

CNS-derived NSCs and ESNPCs have been shown to replace neural populations following SCI. NSCs and ESNPCs can improve remyelination and integrate into axonal pathways, promoting functional recovery (Vroemen et al., 2003, Keirstead et al., 2005, Karimi-Abdolrezaee et al., 2006, Hooshmand et al., 2009). Early work involving murine NSCs in PLGA scaffolds demonstrated that the combination could enhance functional recovery (Teng et al., 2002). Brain-derived NSCs seeded in guidance channels in chitosan scaffolds increased the tissue bridge area following
complete transection (Nomura et al., 2008). Recently, human NSCs in PLGA scaffolds were transplanted into a non-human primate model of SCI (Pritchard et al., 2010). However, sufficient animals for statistical analysis were not used, so the outcomes were inconclusive. Following cortical impact TBI, laminin-based scaffolds containing NSCs improved spatial learning. Improvements in cognitive function where not observed in scaffold only or NSC only treatment groups (Tate et al., 2009). The transition of NSCs into the clinical setting, however, is hindered by limited differentiation of NSCs into neurons in vivo and lack of appropriate donor tissue for human NSCs (Cattaneo and McKay, 1990).

ESNPCs may provide an alternative to NSCs. When transplanted in fibrin scaffolds containing NT-3 and platelet-derived growth factor (PDGF), ESNPCs enhanced functional recovery in a sub-acute hemisection model (Johnson et al., 2010a). Prolonged release of growth factors, however, increased tumor formation from transplanted ESNPCs. Methods for purification of cell populations prior to transplant are necessary before the full utility of ESNPCs can be realized. Induced pluripotent stem cell-derived NSCs may provide an alternative to ESNPCs, however, more work is needed to increase purity and determine the utility of this cell type.

1.5.2 Cell Transplantation and Growth Factor Delivery Combinations

In the developing CNS, neurotrophic factors promote the directed growth and survival of many types of neurons. The introduction of neurotrophins to the injured spinal cord can promote neuronal survival and enhance regeneration of specific ascending and descending axonal pathways. Notably, BDNF promotes growth of
rubrospinal, raphespinal, cerulospinal and reticulospinal pathways while NT-3 elicits sprouting and growth of corticospinal and dorsal column sensory axons (for a complete review see (Lu and Tuszynski, 2008)) (Grill et al., 1997, Ye and Houle, 1997, Menei et al., 1998, Bradbury et al., 1999). NGF and GDNF also support growth of regenerating axons, however, their potential for promoting aberrant growth of pain-associated nociceptive spinal axons may reduce their desirability for SCI (Tuszynski et al., 1994, Blesch and Tuszynski, 2003, Ramer et al., 2003). Combining neurotrophic factor delivery with cell transplantation or biomaterial scaffolds may provide synergistic effects to improve functional recovery.

Coupling cell transplantation with neurotrophic factor delivery may enhance repair following SCI. OPCs modified to express ciliary neurotrophic factor survived to a greater extent compared to unmodified OPCs following transplantation into the contused spinal cord (Cao et al., 2002). Survival correlated with enhanced remyelination of spared axons and recovery of locomotor function. Co-transplantation of NT-3 expressing Schwann cells with NSCs improved locomotor recovery over transplants of unmodified Schwann cells and NSCs (Guo et al., 2007). Axonal growth is commonly reported in response to cellular delivery of neurotrophins (Grill et al., 1997, Menei et al., 1998, Golden et al., 2007, Koda et al., 2007); however, functional recovery is variable and often modest. Many cells endogenously express neurotrophins, thereby reducing the effect of additional secretion on locomotor recovery. Coupling enhanced neurotrophin release with other cell-type specific functions, such as remyelination by OPCs, can improve the utility of these combination therapies.
1.6 Concluding Remarks

In this introduction, the promise of cell replacement therapies, especially ES cell-based therapies, was demonstrated through review of published literature. The potential and associated caveats of combination therapies involving biomaterial scaffolds, growth factor delivery, and cell transplantation was also discussed. The following studies were developed to overcome the most apparent pitfalls of ES cell-based cell replacement therapies: tumorigenicity and heterogeneity of transplanted cell populations.

In the first study, high purity pMNs were obtained and characterized. A new mouse ES cell line (P-Olig2) was developed in collaboration with the laboratory of Dr. David Gottlieb. In this cell line, expression of the puromycin resistance enzyme was driven by gene regulatory elements of the Olig2 in the P-Olig2 cell line. Puromycin exposure was used to isolate pMN cells from the heterogeneous cell culture. The resulting cell populations were characterized for pMN enrichment. This study evaluated the feasibility of antibiotic selection for purification of ES cell-derived populations.

In the second study, high purity pMNs were assessed for their ability to survive and differentiate in fibrin scaffolds. The effect of two different growth factor combinations (NT-3 and GDNF or NT-3 and PDGF) on cell differentiation with or without the HBDS was studied as growth factors have been shown to modulate differentiation of ESPNCs. The ability of in vitro cultures to predict in vivo differentiation was examined following a 2 week transplantation study in a sub-acute dorsal hemisection model of SCI. The sub-acute model was chosen as it has been previously shown to permit greater cell survival. This study examined the ability of
purified pMN populations to differentiate and survive within combination therapies for SCI.

Finally, the third study focus on the development of high-purity ES cell-derived motoneurons. High purity motoneuron populations are difficult to obtain, especially from ES cells. Many methods have been developed for isolating motoneurons from fetal spinal tissue but are not suited well for ES cell-derived motoneurons. Small enhancer elements for the motoneuron transcription factor Hb9 were used to drive puromycin resistance in motoneurons with subsequent purification by puromycin exposure. The ability to generated pure mature neuronal ES cell-derived cultures was investigated.
Chapter 2*

Transgenic Enrichment of Mouse Embryonic Stem Cell-derived Progenitor Motor Neurons

2.1 Abstract

Embryonic stem (ES) cells hold great potential for replacing neurons following injury or disease. The therapeutic and diagnostic potential of ES cells may be hindered by heterogeneity in ES cell-derived populations. Drug selection has been used to purify ES cell-derived cardiomyocytes and endothelial cells but has not been applied to specific neural lineages. In this study we investigated positive selection of progenitor motor neurons (pMNs) through transgenic expression of the puromycin resistance enzyme, puromycin N-acetyl-transferase (PAC), under the Olig2 promoter. The protein-coding region in one allele of Olig2 was replaced with PAC to generate the P-Olig2 cell line. This cell line provided specific puromycin resistance in cells that express Olig2, while Olig2− cells were killed by puromycin. Positive selection significantly enriched populations of Olig2+ pMNs. Committed motoneurons (MNs) expressing Hb9, a common progeny of pMNs, were also enriched by the end of the selection period. Selected cells remained viable and differentiated into mature cholinergic MNs and oligodendrocyte precursor cells. Drug resistance may provide a scalable and inexpensive method for enriching desired neural cell types for use in research applications.

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2.2 Introduction

In most neurological disorders, neurogenesis is insufficient to replenish lost neuronal populations. Endogenous stem cell populations are hindered by limited numbers, variable proliferation in response to disease, and in some cases, differentiation into glia rather than neurons (Baker et al., 2004, Yang et al., 2006, Meletis et al., 2008, Barnabe-Heider et al., 2010). ES cells can be differentiated into specific neuronal subtypes and may be useful for cell replacement strategies in the central nervous system (Sonntag et al., 2007). Transplantation of ES cell-derived dopaminergic neurons and cholinergic MNs has been shown to promote partial recovery from Parkinson’s-like symptoms and spinal cord injury, in rodent models (Roy et al., 2006, Erceg et al., 2010). Heterogeneous populations arising from differentiation of ES cells, however, currently limit the efficacy of such treatments (Gogel et al., 2011). Strategies for controlled differentiation of ES cells and the subsequent enrichment ES cell-derived cells types are therefore critical to the translation of ES cell-based therapies into a clinical setting.

Directed differentiation of ES cells into spinal MNs can be achieved following exposure to retinoic acid (RA) and sonic hedgehog (Shh) (Wichterle et al., 2002, Wichterle and Peljto, 2008). During this process, ES cells first differentiate into pMNs expressing the basic helix-loop-helix transcription factor Olig2 (Mizuguchi et al., 2001, Novitch et al., 2001). These cells can commit to the MN fate by downregulating Olig2 and expressing the homeodomain (HD) transcription factors Islet 1 (Isl1) and Hb9, also known as Mnx1 (Pfaff et al., 1996, Arber et al., 1999, Mizuguchi et al., 2001, Novitch et al., 2001). Despite optimization, differentiation protocols for pMNs result in a
heterogeneous population of cells including other ventral spinal progenitor cells (Wichterle et al., 2002). Hb9<sup>+</sup>-committed MNs compose only 15-50% of the total culture after differentiation of ES cells (Deshpande et al., 2006, Wichterle and Peljto, 2008). Low-purity cultures give rise to multiple types of spinal interneurons, therefore subsequent enrichment may be necessary (Deshpande et al., 2006).

Greater pMN purity can be obtained by fluorescence-activated cell sorting (FACS) of a transgenic ES cell line that expresses GFP under the Olig2 gene regulatory elements (GRE) (Xian et al., 2003, Xian and Gottlieb, 2004, Xian et al., 2005). This method, however, requires expensive equipment and must be performed at a centralized facility, risking contamination. Gradient centrifugation can enrich spinal MNs from the mouse embryonic lumbar spinal cord and human ES cells, but has not been optimized for mouse ES cell-derived MNs (Wada et al., 2009, Wiese et al., 2010). Transgenic selection may provide a low-cost alternative and can be performed directly in the culture dish. Puromycin resistance through expression of the enzyme PAC has been shown to allow enrichment of ES cell-derived cardiomyocytes and endothelial cells in transgenic lines (Marchetti et al., 2002, Kolossov et al., 2006, Anderson et al., 2007, Kim and von Recum, 2009), but has not been used to enrich specific neural populations.

In this study, we investigated whether transgenic selection could help to enrich low-purity populations that commonly result from pMN differentiation protocols. We generated a new heterozygous “knock in” mouse ES cell line (P-Olig2) where the protein-coding region in one allele of Olig2 was replaced with PAC, allowing for positive selection of Olig2<sup>+</sup> pMNs during the differentiation. Olig2 expression was analyzed during directed differentiation of ES cells into pMNs using the Shh signaling
agonist, purmorphamine (Wu et al., 2004, Sinha and Chen, 2006). Puromycin-treated cells were assessed for expression of pMN-specific markers and differentiation into pMN progeny, including MNs and oligodendrocytes. This study demonstrates the first use of puromycin resistance for positive selection of a specific population of neural progenitor cells.

2.3 Materials and Methods

2.3.1 Embryonic Stem Cell Culture

The RW4 ES cell line was used to generate and characterize the P-Olig2 cell line. Olig2 expression was characterized using the G-Olig2 ES cell line that expresses GFP under the control of the Olig2 GRE (Xian et al., 2003, Xian and Gottlieb, 2004, Xian et al., 2005). ES cells were grown in complete media consisting of Dulbecco’s modified Eagle’s Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (Invitrogen), 10% fetal bovine serum (Invitrogen), 10 µM thymidine (Sigma, St. Louis, MO), and 30 µM of each of the following nucleosides: adenosine, cytosine, guanosine, and uridine (Sigma). Cells were passaged at a 1:5 ratio every 2 days and seeded on a new T25 flask coated with a 0.1% gelatin solution (Sigma). After seeding, 1000 U/ml leukemia inhibitory factor (LIF; Millipore, Billerica, MA) and 100 µM β-mercaptoethanol (BME; Invitrogen) were added to the media to maintain the undifferentiated state of the ES cells without the need for a feeder cell layer.
2.3.2 Generation of P-Olig2 ES Cells

The P-Olig2 cell line was generated from the RW4 line. Approximately $1 \times 10^7$ RW4 ES cells were resuspended in electroporation buffer with 10-15 µg of ScaI-linearized P-Olig2 targeting vector. The targeting vector was constructed from a Gateway-compatible plasmid (pStartK) incorporating the Olig2 locus with the Olig2 open reading frame replaced by a dual resistance cassette consisting of from 5’ to 3’: Asc1 site, Kozak sequence, puromycin cassette with bgp polyA signal (PKO-Select Puro, Agilent Genomics, Santa Clara, CA), floxed phosphoglycerate kinase I promoter driving the neomycin phosphotransferase gene (PGK-neo) with bgp polyA signal, and AscI site (Kozak, 1986, Thomas and Capecchi, 1987, Wu et al., 2008). Cells were electroporated using an Amxa nucleofector II (Lonza, Basel, Switzerland) at 0.23kV and 960µF in a 0.4 cm cuvette (Bio-Rad, Hercules, CA). Following electroporation, cells were seeded on gelatin coated 10cm dishes for 24 hours then dosed with G418 (200 µg/ml, Invitrogen) and 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU; 100 nM, Movarek Biochemicals, Brea, CA) for positive and negative selection respectively. After 8 days, resistant clones were picked and seeded on a mouse embryonic fibroblast STO monolayer in individual wells of a 96 well plate to promote growth of low density ES cell clones. Clones were screened for targeting events by PCR using standard methods. Targeted clones were detected and further characterized.

2.3.3 Southern Hybridization

Genomic DNA was isolated from RW4 and P-Olig2 ES cells using the ArchivePure DNA Cell/Tissue and Tissue Kit (5 Prime, Gaithersburg, MD). DNA-
binding proteins were digested with Proteinase K. Genomic DNA was digested with 200 U HindIII or 50 U SpeI overnight at 37°C. Restriction enzymes were re-applied for an additional hour then DNA was ethanol precipitated. DNA restriction fragments were separated by electrophoresis. DNA was transferred to a Hybond-XL (GE Healthcare Biosciences, Piscataway, NJ) membrane and crosslinked using a UV Stratalinker 2400 (Stratagene). DNA probes were prepared with the Rediprime Kit (GE Healthcare Bioscience) and [³²P] dCTP (Perkin-Elmer, Waltham, Massachusetts). Probes were purified using illustra ProbeQuantG-50 columns (GE Healthcare Biosciences). Blots were hybridized in Rapid hybe (GE Healthcare Biosciences) for two hours at 65°C, then washed and visualized by autoradiography.

2.3.4 Cre-excision

To remove the floxed PGK-neo resistance cassette, 2x10⁶ ES cells were transfected with 5 µg of Cre recombinase expressing plasmid (p1411, gift of Tim Ley, Washington University). Removal of the PGK-neo cassette was confirmed using junction PCR. Cre-excised clones were further subcloned and re-validated by PCR and neomycin sensitivity.

2.3.5 Live Dead Assay

Live/Dead reagent (Invitrogen) consisting of calcien-AM and ethidium homodimer was used to visualize live and dead cells, respectively. Wells were washed with Dulbecco’s PBS and incubated with 1x Live/Dead reagent for 30 min at room
temp. Fluorescent images were captured using MetaVue image analysis software (Molecular Devices, Sunnyvale, CA) and a Nikon TE200S fluorescence microscope.

2.3.6 pMN Differentiation

For pMN induction, ES cells were exposed to RA (Sigma) and purmorphamine (EMD, Gibbstown, NJ) in a 2/4 + differentiation protocol. One million ES cells were aggregated into embryoid bodies (EBs) in 100-mm Petri dishes coated with a 0.1% agar solution in DFK5 media consisting of DMEM:F12 base media (Invitrogen) supplemented with 5% knockout serum replacement (Invitrogen), 50 µg/ml apo-transferrin (Sigma), 50 µM non-essential amino acids (Invitrogen), 5 µg/ml insulin (Sigma), 30 nM sodium selenite (Sigma), 100 µM β-mercaptoethanol, 5 µM thymidine, and 15 µM of the following nucleosides: adenosine, cytosine, guanosine, and uridine. EBs were allowed to form for 2 days in the absence of inducing factors, then split 1:5 and transferred to 6-well plates coated with 0.1% gelatin solution. Once plated, the EBs were grown for an additional 4 days in DFK5 supplemented with 2 µM RA and 250 nM – 1.5 µM purmorphamine. Media was changed every 2 days. In selected cultures, 2 ng/ml puromycin (Sigma) was added during the final 2 days of differentiation.

2.3.7 Quantitative Real Time Polymerase Chain Reaction

The relative expression level of progenitor cell transcription factors and markers for MN differentiation were assessed using quantitative real-time polymerase chain reaction (qRT-PCR). EBs were lysed with Trizol reagent (Invitrogen) and RNA was isolated using an RNeasy kit (Qiagen, Germantown, MD). Isolated RNA was used to
synthesize cDNA for qRT-PCR analysis using the TaqMan 2-Step RNA-to-CT Mini Kit (Applied Biosystems, Carlsbad, CA). TaqMan Gene Expression Assays (Table 1, Applied Biosystems), TaqMan Gene Expression Master Mix (Applied Biosystems) and cDNA were combined and qRT-PCR was performed using an Applied Biosystems 7000 thermocycler with the following PCR protocol: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. 6-carboxyfluorescein (FAM) fluorescent detection occurred during each 72°C cycle. Relative mRNA expression was reported as the number of cycles necessary for fluorescent intensity to increase exponentially, referred to as the threshold cycle \((Ct)\). All target genes were normalized to ß–actin to account for differences in total mRNA content. Expression in EBs induced with 2 µM RA and 250 nM, 500 nM, or 1 µM purmorphamine was determined using the comparative \(\Delta C_t\) method, with EBs receiving neither RA nor purmorphamine serving as the control group (Schmittgen and Livak, 2008). Results are reported as a fold difference in relative RNA expression over control EBs (n=3 for each condition).

2.3.8 Flow Cytometry

Differentiated ES cell cultures were stained immediately following the 2/4+ treatment protocol for flow cytometry analysis. Cultures were dissociated with trypsin-EDTA (0.25%; Invitrogen) for 15 min and triturated to form single cell suspensions. Excess volume of complete media was added to quench the trypsin, and cells were centrifuged for 5 min at 230xg. The media was aspirated and cells were fixed with 1% paraformaldehyde (Sigma). After fixation, the cells were permeabilized with 0.5% saponin (Sigma) solution for 20 min, and then blocked in 0.1% saponin solution.
containing 5% normal goat serum (NGS; Sigma). Cell suspensions were then incubated for 30 min in 0.1% saponin solution containing 2% NGS and one of the following primary antibodies: Nestin (Iowa Hybridoma Bank; 1:10), Isl1 (Iowa Hybridoma Bank; 1:50), Hb9 (MRN2; Iowa Hybridoma Bank; 1:25), and Olig2 (Millipore; 1:500). Cells were washed with PBS and appropriate Alexa Fluor secondary antibodies (1:200; Invitrogen) diluted in 0.5% saponin with 2% NGS were applied for 30 min. Finally cells were washed with PBS and incubated with Hoechst (1:1000; Invitrogen) for 5 min.

Stained cell suspensions were analyzed using a Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For each group, 10,000 events were recorded. Subsequent analysis was performed using FloJo software (FloJo, Ashland, OR). Prior to population gating, debris was removed based on forward scatter versus side scatter and Hoechst fluorescence versus forward scatter plots. Flow cytometry control groups, consisting of cells stained with the secondary antibody only, were used to determine quadrant population gating parameters. Flow cytometry results are presented as the percentage of cells staining positive for each marker out of the total live cell population.

2.3.9 Immunocytochemistry

Cell distribution and identity was assessed in differentiated cultures using immunocytochemistry (ICC). Cell cultures were fixed with 1% paraformaldehyde for 30 min then permeabilized in 0.01% Triton X (Sigma) for 15 min. The cells were blocked with 5% NGS for 1 hour at 4°C and incubated overnight at 4°C in 2% NGS solution with one or more of the following primary antibodies: Oct 4 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), Olig2 (1:500), Isl1 (1:20), Hb9 (1:20), Nestin (1:10),
Neurofilament (NF, DSHB, 1:25), choline acetyl transferase (ChAT, Millipore, 1:400), oligodendrocytes (RIP, Millipore, 1:5000), and oligodendrocyte marker 4 (O4, Millipore, 1:500). Primary antibody staining was followed by 3 washes in an excess volume of PBS for 15 min each. Each culture was then stained with the appropriate Alexa Fluor secondary antibodies (Invitrogen) for 1 hour at 4°C followed by an additional 3 washes in PBS. Cell nuclei were stained with the nuclei binding dye Hoechst (1:1000). Fluorescent images were captured using a MICROfire camera attached to an Olympus IX70 inverted microscope. Images were analyzed using Image Pro Express (Media Cybernetics, Silver Spring, MD).

2.3.10 Undifferentiated ES Cells

To quantify the occurrence of Oct4+ undifferentiated stem cells following the 2'4' differentiation protocol, P-Olig2 cultures were fixed and stained with the Oct4 antibody. EBs containing at least one Oct4+ nuclei were counted and divided by the total number of EBs to determine the fraction of EBs containing undifferentiated stem cells. At least 50 EBs per sample were assessed for Oct4 expression.

2.3.11 Extended Differentiation of pMN

For pMN differentiation, 24-well plates were pre-coated with 0.01% poly(ornithine) solution (Sigma) then coated with 0.01 mg/mL laminin solution (Invitrogen) overnight. Induced 2'4' EBs were dissociated and plated at a density of 100,000 cells/ml in DFK5 media. After 5 days, media was replaced with Neurobasal
media (Invitrogen) supplemented with 0.1% bovine serum albumin (Sigma) and 2% B27 (Invitrogen). Cells were allowed to differentiate an additional 9 days.

2.3.12 Statistical Analysis

For qRT-PCR and flow cytometry analyses, 3 replicates of each condition were performed. Statistical analysis was performed in Statistica software (version 5.5; StatSoft; OK). Multiple comparisons statistics were accomplished using Scheffe’s post hoc test for analysis of variance (ANOVA) with a 95% confidence level. Values are reported as the mean plus or minus standard deviation.

2.4 Results

2.4.1 Olig2 expression during differentiation of ES Cells

To determine the effect of Shh signaling levels on directed differentiation of ES cells into pMNs, we analyzed mRNA levels in response to increasing concentrations of purmorphamine, a Shh agonist, using quantitative real time (RT)-PCR. ES cells were exposed to 2 µM retinoic acid (RA) and 250 nM, 500 nM, or 1 µM purmorphamine. Relative mRNA levels were analyzed at the end of the 2/4+ differentiation protocol and were compared to control cells that did not receive RA or purmorphamine (n=3 for all conditions). Increasing the purmorphamine concentration from 250 nM to 1 µM led to downregulation of Dbx2 and Irx3, two transcription factors found in p1 and p2 progenitor (more dorsal) domains, respectively (Figure 2.1, Figure 2.2A). The mRNA levels for Pax6, which is expressed in the p1, p2, and pMN domains, did not change.
with concentration. Nkx2.2 mRNA levels were too low for detection even at the highest concentration of purmorphamine (data not shown). Olig2 expression significantly increased with exposure to 1 µM purmorphamine compared to 250 nM and 500 nM purmorphamine (Figure 2.2B). HD transcription factors Isl1 and Hb9 expressed during commitment of pMNs to the MN fate were upregulated with 1 µM purmorphamine, similar to Olig2. Finally, 1 µM purmorphamine led to an increase in mRNA for choline acetyltransferase (ChAT), an enzyme found specifically in mature MNs.

Figure 2.1 Schematic of transcription factors expressed in spinal progenitor domains. Ventral-to-dorsal gradient of sonic hedgehog (Shh) and relative position of spinal progenitor domains in the ventral neural tube are shown on the left. The pattern of transcription factor expression in each domain is shown on the right.
2.4.2 Heterogeneity in differentiated cultures

To characterize the heterogeneity of the cell population resulting from differentiation of ES cells, we utilized a transgenic ES cell line expressing GFP under the Olig2 GRE, G-Olig2 (Xian and Gottlieb, 2004). GFP fluorescence can persist for several days after transcription, allowing for identification of pMNs and their recent progeny during differentiation. By visual inspection of GFP fluorescence, pMNs could be easily separated from other spinal progenitor cells that differentiate into spinal interneurons rather than MNs.

G-Olig2 ES cells were differentiated using 1 µM purmorphamine and 2 µM RA and analyzed by flow cytometry. At the end of the 2/4+ differentiation protocol, 61.6 ± 4.5% of cells expressed GFP (n = 3). This percentage was approximately 3-fold higher.
than expression of Olig2 found with traditional antibody staining, suggesting that GFP fluorescence persists in recent pMN progeny as expected. GFP cells were consistently found migrating away from EBs and displayed a broad flat morphology typical of astrocytes or astrocyte precursor cells (ASPs) (Figure 2.3 D-F; white arrows). These cells may originate from the p2 progenitor domain that gives rise to ASPs in vivo (Muroyama et al., 2005). Additional GFP cells were present within EBs with a similar morphology to Olig2+ pMNs and may represent progenitor cells from adjacent progenitor domains (white asterisk).

2.4.3 Generating the P-Olig2 Cell Line

The P-Olig2 cell line was generated using a targeting vector with a resistance cassette in the open reading frame of the Olig2 gene surrounded by two regions homologous to the Olig2 locus. RW4 ES cells were electroporated with the P-Olig2 targeting vector and homologous recombination occurred as illustrated in (Figure 2.4A). To confirm targeted insertion, novel junctions were detected using short arm junction PCR. Successful integration resulted in a 2.1 kb fragment spanning from inside the targeting construct into neighboring genomic DNA (Figure 2.4B). The addition of the resistance cassette increased the distance between Hind III sites within the Olig2 locus from 4.8 kb to 6.7 kb as observed by Southern analysis (Figure 2.4C). Only the 4.8kb band was observed in control RW4 cells while both bands were observed in the P-Olig2 cell line. The appearance of both bands confirms that only one of the two Olig2 loci was targeted. Following insertion, the floxed PGK-neo cassette was excised with Cre
Figure 2.3 Heterogeneity following directed differentiated of G-Olig2 ESCs. (A): Phase contrast image of differentiated G-Olig2 ESCs following exposure to purmorphamine and RA. (B): Expression of green fluorescent protein (GFP) in (A) showing cells that have expressed Olig2 during differentiation. (C): Corresponding Hoechst staining showing cell nuclei. Scale bars = 100 µM. (D-F) Close inspection of GFP cells (white arrows) with astrocyte-like morphology that did not differentiate into pMNs expressing Olig2. Additional GFP cells with progenitor-like morphology were found within EBs (white asterisk). Scale bars = 50 µM.
recombinase as confirmed using PCR by a new 350 bp band (Figure 2.4D). The final cell line contained a promoter-less PAC cassette driven by the Olig2 GRE (Figure 2.5A).

To determine the specificity of PAC expression, P-Olig2 and RW4 ES cells were differentiated into pMNs, and the expression of Olig2 and PAC mRNA was assessed using PCR. Olig2 and PAC mRNA was not observed or present at very low levels in ES cells and ES cells aggregated into EBs (Figure 2.5B). Following the 2/4+ differentiation protocol, Olig2 mRNA levels were elevated in both P-Olig2 and RW4 cell lines. PAC mRNA, however, was specific to the P-Olig2 cell line. Specificity of PAC expression in the P-Olig2 cell line was confirmed by sensitivity to puromycin. Few P-Olig2 ES cells survived following exposure to puromycin for 48 hrs (Figure 2.5C) since the Olig2 gene is off or expressed at very low levels. In contrast, many viable cells were observed in cultures of P-Olig2 ES cells differentiated into pMNs, which express Olig2 (Figure 2.5D). Widespread cell death was still observed in the latter group, suggesting the presence of Olig2 cells that do not express PAC and are sensitive to puromycin.

2.4.4 Enrichment of pMNs using the P-Olig2 Cell Line

To determine whether transgenic selection enriched the cell population for pMNs and committed MNs, P-Olig2 cells were differentiated using 2 µM RA and 1.5 µM pumorphamine with a 2/4+ differentiation protocol. Puromycin was added during the last two days to select for Olig2+ pMNs (Figure 2.6A). Control groups consisted of RW4 and P-Olig2 cells not receiving puromycin. Similar to G-Olig2 cell cultures, broad Olig2 cells were observed migrating out of the EB in the control P-Olig2 group (Figure
Figure 2.4 Knock-in to replace Olig2 ORF with PAC gene. (A): Olig2 gene is a schematic of the Olig2 gene. The smaller black boxes represent the two exons of the Olig2 gene with the ORF (large black box) located in the second exon. The 5' probe and H (HindIII) sites used for Southern blots are indicated. P-Olig2 vector shows the targeting vector with the PAC and floxed neo cassette in place of the ORF. P-Olig2-neo is the predicted knock-in product with the predicted 6.7Kb HindIII fragment and the 2.1 Kb junction PCR product shown. P-Olig2 shows the engineered gene after Cre-excision of the floxed neo cassette. (B): Junction PCR of control RW4 cells and targeted cells. Amplified DNA is absent in the RW4 cells but present in the targeted (P-Olig2) cells. (C): Southern blots- RW4 parental cells have native 4.8 Kb HindIII band; targeted (POlig2) cells have additional 6.7 Kb band. (D) Cre-excision of neo cassette- PCR reactions across neo cassette show amplified DNA in Cre+ cells but not in untreated (Cre-) cells.
In cultures with puromycin, these cells were not present. Cellular debris localizing to the same region suggests that puromycin-induced cell death occurred in Olig2 cells. The majority of surviving cells had a progenitor-like morphology and expressed Olig2 or Hb9.

To assess cell differentiation in control and selected cultures, cells were stained at the end of the differentiation protocol with phenotype-specific markers and analyzed using flow cytometry. Puromycin selection did not affect the distribution of Nestin+ cells, suggesting that the majority of cells were neural cells. The percentage of Olig2+ cells increased significantly from 21.9 ± 5.9% to 48.7 ± 7.3% when selected with puromycin (Figure 2.7A-B). Hb9+ populations were enriched from 20.6 ± 7.9% to 58.5 ± 1.5%. Similar enrichment was observed for Isl1. No significant differences were found for any marker between the RW4 and control P-Olig2 (non-selected) groups.

2.4.5 Undifferentiated Stem Cells

To determine the effect of puromycin exposure on Oct4+ undifferentiated stem cells, cultures were fixed and analyzed following immunocytochemistry (ICC). The overall percentage of cells expressing the pluripotent stem cell marker Oct4 following the 2/4+ differentiation was low (<1%) in control and selected groups (data not shown). The fraction of 2 /4+ EBs containing at least one Oct4+ nuclei was compared between unselected and selected P-Olig2 pMNs on day 6. Oct4+ cells were commonly found in small groups and were limited to unselected P-Olig2 cultures (Figure 2.8A). Approximately 10% of EBs contained undifferentiated stem cells in the absence of puromycin (Figure 2.8B). Oct4+ nuclei were not observed in any of the cultures selected with puromycin.
Figure 2.5 PAC and Olig2 expression in the P-Olig2 cell line (A): Schematic of the PAC cassette driven by the native Olig2 GRE. (B): Olig2 and PAC mRNA expression in P-Olig2 and RW4 cell lines. Expression was analyzed in ESCs, ESCs aggregated into embryoid bodies (EBs), and ESCs differentiated into pMNs using a 2-/4+ treatment protocol. (C): Live/dead assay showing live cells (green) and dead cells (red) following exposure of undifferentiated P-Olig2 and RW4 ESCs to puromycin for 48 hours. (D): Live/dead assay for differentiated P-Olig2 and RW4 EBs selected with puromycin for 48 hours. Fluorescent cell debris was observed in the RW4 group whereas the P-Olig2 group contained whole viable cells.
2.4.6 Extended Differentiation of pMNs

To determine whether targeted replacement of the Olig2 gene impacted the ability of ES cell-derived pMNs to terminally differentiate, we cultured puromycin selected P-Olig2 cells for two weeks on laminin-coated wells. Following differentiation, cells were assessed for expression of ChAT and neurofilament (NF) using ICC to identify mature MNs. Cultures were also stained for oligodendrocyte marker 4 (O4) and RIP to identify oligodendrocytes, another common progeny of pMNs. Groups of ChAT⁺/NF⁺ neurons were abundant throughout the culture (Figure 2.9). Oligodendrocytes expressing O4 and RIP were also present (Figure 2.9). These results demonstrate that cells surviving the puromycin selection differentiate into neurons and oligodendrocytes, the expected progeny of Olig2⁺ pMNs.

2.5 Discussion

Current protocols for differentiation of mouse ES cells are often hindered by low efficiencies. Directed differentiation may be lead to heterogeneous ES cell-derived populations that must be further purified to obtain the desired lineages prior to cell culture studies or transplantation. In this study, we demonstrate that positive selection of Olig2⁺ pMNs through transgenic expression of the puromycin resistance enzyme PAC can provide a simple method for enrichment of pMNs.

Purmorphamine exhibited a dose-dependent effect on pMN gene expression during directed differentiation of G-Olig2 ES cells. Greater concentrations of
Figure 2.6 Positive selection of P-Olig2 ESCs differentiated using a 2/4+ differentiation protocol. (A): Schematic showing 2/4+ differentiation protocol of ESCs. Puromycin was added from day 4 and remained till day 6. (B): Immunocytochemistry analysis of P-Olig2 ESCs following differentiation and puromycin selection. Olig2+ and Hb9+ cells compose a small fraction of the entire population in the absence of puromycin. Following puromycin treatment, the majority of cells expressed either Olig2 or Hb9. (C): Nestin expression in control and selected P-Olig2 cultures. The majority of cells express nestin.
Figure 2.7 Flow cytometry results following selection. (A) Flow cytometry histograms and gating. Solid black histograms represent secondary antibody controls. (B): Flow cytometry analysis of RW4, non-selected P-Olig2, and puromycin selected P-Olig2 ESCs following differentiation. * indicates p < 0.05 for that marker compared to unselected P-Olig2 group. Scale bars = 100 µM.

Figure 2.8 Oct4 expression in P-Olig2 cells following the 2 / 4+ differentiation protocol. (A): A small group of Oct4+ nuclei in an unselected, control P-Olig2 EB. (B): Fraction of EBs containing Oct4+ nuclei in control and selected P-Olig2 cultures. * indicates p < 0.05 compared to unselected P-Olig2 group.
purmorphamine led to a significant increase in \textit{Olig2} expression. Conversely, expression of \textit{Dbx2} and \textit{Irx3} (more dorsal transcription factors) were reduced with increasing concentrations of purmorphamine. \textit{Nkx2.2}, which is expressed in the more ventral p3 progenitor domain, was not detected in any of the conditions tested. Based on the gene expression data, directed differentiation appears to favor pMNs at the highest concentration of purmorphamine tested. However, this condition still resulted in a mixed cell population with nearly 40\% of cells not expressing \textit{Olig2}. This undesired population may include cells from neighboring progenitor domains that have been previously observed following directed differentiation of ES cells into pMNs (Wichterle et al., 2002). Differentiation of ES cells into cells from multiple spinal progenitor domains is potentially due to overlapping dependency on Shh signaling in the ventral neural tube or our inability to precisely control localized concentration of purmorphamine over the duration of the experiment. Furthermore, variations in the responsiveness of each cell to Shh signaling may attribute to heterogeneity.

Positive selection of cardiomyocytes and endothelial cells through puromycin resistance has been previously shown using randomly inserted resistance cassettes containing a cell-type specific promoter (Marchetti et al., 2002, Kolossov et al., 2006, Kim and von Recum, 2009). By knocking in PAC expression, we preserve regulatory mechanisms for the native \textit{Olig2} gene. Expression of PAC in the final P-\textit{Olig2} cell line recapitulated expression of \textit{Olig2} in the native allele. Specificity of the PAC cassette driven by the native \textit{Olig2} GRE was shown by puromycin sensitivity. Cell death was induced in puromycin treated P-\textit{Olig2} ES cells within 48 hours which is similar to the
time-course previously described for puromycin-induced cell death (Watanabe et al., 1995). Only when P-Olig2 ES cells were differentiated into pMNs using the 2/4+ differentiation protocol did cells remain viable following puromycin treatment. Cell death was still observed in differentiated ES cell cultures suggesting that only cells having expressed Olig2 show resistance. Consistent with this hypothesis, puromycin treatment removed all broad flat cells previously identified as Olig2+. In addition, puromycin killed all Olig2+/Oct4+ undifferentiated stem cells. The majority of remaining viable cells were positive for the transcription factors Olig2 or Hb9. These results demonstrate selective resistance in pMNs and their progeny.
Differentiation of non-selected P-Olig2 ES cells was similar to RW4 ES cells for all markers tested in this study. Substituting PAC for one copy of the Olig2 gene did not appear to alter differentiation of P-Olig2 ES cells into pMNs or commitment of pMNs to the MN fate. Following puromycin treatment, Olig2+ pMNs were significantly enriched. High purity pMNs cultures were not obtained in this study; perhaps this is due to the persistence of the PAC enzyme in committed Hb9+ MNs. Committed MNs were enriched nearly 3-fold and constituted the majority of cells at the end of the selection. This population may become further enriched as additional Olig2+ pMNs commit to the MN fate. The distribution of Nestin+ cells was not affected by puromycin treatment, suggesting that the majority Olig2- cells killed by puromycin were neural lineage cells. These cells could be other spinal progenitor cells that express nestin.

The long-term effects of PAC knock-in and puromycin exposure were assessed through differentiation of puromycin-treated pMNs on laminin. Following a two week differentiation period, ChAT+/NF+ MNs and O4+/RIP+ immature oligodendrocytes were observed. These results demonstrate long-term viability of enriched pMNs and committed MNs, suggesting no persistent effects of puromycin exposure.

Oligodendrocyte development from pMNs is dependent on continuous Olig2 expression (Xian and Gottlieb, 2004), and does not appear to be disturbed by the PAC knock-in. Both MNs and oligodendrocytes have been transplanted for treatment of SCI (Keirstead et al., 2005, Sharp et al., 2010) and are potential target populations for cell replacement strategies in other neurological disorders including amyotrophic lateral sclerosis and multiple sclerosis. Additional enrichment strategies can be employed to purify individual populations of MNs or oligodendrocytes.
Many studies in developmental biology, drug screening, and regenerative medicine can benefit from high purity ES cell-derived cell populations. This study demonstrates the use of puromycin resistance to select for a well-defined set of neural progenitor cells following directed differentiation of ES cells. The methods utilized in this study can be applied to other neural cell types to generate high-purity populations that can accelerate the use of ES cell-based research in scientific and clinical settings.
Chapter 3

Purified Progenitor Motor Neuron Cell Transplants Survive and Differentiate in a Sub-acute Model of Spinal Cord Injury

3.1 Abstract

Embryonic stem (ES) cells hold great potential for producing cell types that can be used in cell replacement therapies following spinal cord injury (SCI). Undifferentiated ES cells, however, persist following differentiation and can lead to tumor formation when transplanted into the injured cord. Creating a more favorable transplantation environment through the use of tissue-engineered biomaterial scaffolds may enhance tumor formation by increasing proliferation of undesired cell types. Methods for purification of ES cell-derived neural populations are necessary to recognize the full utility of combination therapies involving biomaterials and ES cell-derived transplants. We have previously developed a method for enriching ES cell-derived progenitor motoneurons (pMNs) differentiated from mouse ES cells via antibiotic selection. The enriched cell populations are devoid of pluripotent stem cells. In this study we demonstrate the survival and differentiation of enriched pMNs within fibrin scaffold in vitro and when transplanted into a sub-acute dorsal hemisection model of SCI.
3.2 Introduction

SCI is a traumatic event that leads to life-long debilitation. Loss of function following injury is associated with severed ascending and descending tracts, cell death, and demyelination of spared axons. Stem cell therapies can improve remyelination and may enhance remodeling of local circuitry following SCI by replacing lost neuronal and oligodendrocyte populations (Cummings et al., 2005, Keirstead et al., 2005, Sharp et al., 2010, Nori et al., 2011). Pre-differentiation of ES cells can be used to obtain various neural cell populations for treatment of SCI (Cummings et al., 2005, Keirstead et al., 2005, Kumagai et al., 2009, Erceg et al., 2010, Rossi et al., 2010, Sharp et al., 2010).

Transplantation of stem cells into the injured cord, however, often leads to poor survival and gliogenic differentiation (Liu et al., 2000, Cao et al., 2001, Cao et al., 2002, Erceg et al., 2010).

Biomaterial scaffolds can improve cell survival, migration, and integration for SCI repair (Teng et al., 2002, Nomura et al., 2008, Patel et al., 2010). Controlled differentiation of ES cell-derived neural progenitor cells (ESNPCs) in vitro into neurons and oligodendrocytes has been shown following encapsulation in tissue-engineered fibrin scaffolds containing a heparin-based growth factor delivery system (HBDS) and specific combinations of growth factors (Willerth et al., 2008). When transplanted into the injured cord, ESNPCs encapsulated in fibrin scaffolds containing the HBDS, neurotrophin-3 (NT3) and platelet-derived growth factor (PDGF) had improved survival two weeks following transplantation compared to ESNPCs transplanted alone or in unmodified fibrin (Johnson et al., 2010b). Even greater neuronal survival and differentiation was observed when growth factors were delivered using the HBDS.
The presence of growth factors, however, promoted rapid proliferation and tumor formation at eight weeks following transplantation (Johnson et al., 2010a). Growth factor delivery from the HBDS further enhanced tumor formation from a small population of undifferentiated stem cells, possibly by creating a more permissive transplantation environment. A small population of undifferentiated pluripotent ES cells that persists following differentiation of ES cells into ESNPCs was hypothesized as the main tumor producing cell type. The tumorigenicity of persistent undifferentiated stem cells may explain terotoma formation observed in several other studies (Bjorklund et al., 2002, Erdo et al., 2003, Amariglio et al., 2009). Methods to purify ES cell-derived populations prior to transplantation may be necessary to reach the full potential of ES cell-derived neural cells in combination with tissue-engineered biomaterial scaffolds.

Recently, we have demonstrated a simple and effective method for purifying ES cell-derived pMNs (McCreedy et al., 2012). Antibiotic resistance driven by the gene regulator elements of the pMN-associated transcription factor, Olig2, allowed for enriched cultures for pMNs and recent progeny of pMNs following directed differentiation of ES cells and concurrent antibiotic selection. The survival and differentiation of high purity pMNs within the injured cord, however, has yet to be assessed. To determine the potential for enriched ES-cell derived pMNs to serve as a potential therapeutic for SCI, we encapsulated cells within fibrin scaffolds containing a combination of NT3 and PDGF, or NT-3 and glial-derived neurotropic factor (GDNF) delivered via the HBDS for two weeks in a sub-acute dorsal hemisection model of SCI. Enriched pMNs survive within fibrin scaffolds and differentiate into motoneurons, oligodendrocytes, and astrocytes with no growth factor associated over proliferation.
3.3 Materials and Methods

3.3.1 Embryonic Stem Cell Culture

P-Olig ES cells were grown in complete media consisting of Dulbecco’s modified Eagle’s Medium (Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (Invitrogen), 10% fetal bovine serum (Invitrogen), 10 µM thymidine (Sigma, St. Louis, MO), and 30 µM of each of the following nucleosides: adenosine, cytosine, guanosine, and uridine (Sigma). Cells were passaged at a 1:5 ratio every 2 days and seeded on a new T25 flask coated with a 0.1% gelatin solution (Sigma). After seeding, 1000 U/ml leukemia inhibitory factor (LIF; Millipore, Billerica, MA) and 100 µM β-mercaptoethanol (BME; Invitrogen) were added to the media to maintain the undifferentiated state of the ESCs without the need for a feeder cell layer.

3.3.2 pMN Differentiation

For pMN induction, ES cells were exposed to retinoic acid (RA) (Sigma) and purmorphamine (EMD, Gibbstown, NJ) in a 2/4+ differentiation protocol (McCreedy et al., 2012). One million ESCs were aggregated into embryoid bodies (EBs) in 100-mm Petri dishes coated with a 0.1% agar solution in DFK5 media consisting of DMEM:F12 base media (Invitrogen) supplemented with 5% knockout serum replacement (Invitrogen), 50 µg/ml apo-transferrin (Sigma), 50 µM non-essential amino acids (Invitrogen), 5 µg/ml insulin (Sigma), 30 nM sodium selenite (Sigma), 100 µM β-mercaptoethanol, 5 µM thymidine, and 15 µM of the following nucleosides: adenosine, cytosine, guanosine, and uridine. EBs were allowed to form for 2 days in the absence of inducing factors (2). EBs were then cultured in DFK5 supplemented with 2 µM RA.
and 1.5 µM purmorphamine for the final four days (4\(^+\)). Media was changed every 2 days. In selected cultures, 4 ng/ml puromycin (Sigma) was added during the final 2 days of differentiation.

### 3.3.3 In Vitro Differentiation

To prepare fibrin scaffolds, fibrinogen (50 mg/mL; EMD) was dissolved in tris-buffered saline (TBS) and dialyzed in 4L TBS overnight. Fibrinogen was sterile filtered and the concentration measured by UV spectroscopy. The final fibrinogen concentration was adjusted to 20 mg/mL with sterile TBS. Fibrin scaffolds (150 µl) were formed by combining 10 mg/ml fibrinogen, 2.5 mM CaCl\(_2\) (Sigma), and 2 NIH units/ml thrombin (Sigma) in individual wells of a 48 well plate. In delivery system (DS) control groups, 62.5 µM heparin (Sigma) and 0.25 mM AT-III peptide were added to each scaffold. In growth factor control groups, scaffolds contained 62.5 µM heparin, 62.5 ng NT3 (Peprotech), and 62.5 ng GDNF (Peprotech) (Hep + NT3 + GDNF) or 10 ng PDGF (Peprotech) (Hep + NT3 +PDGF). For growth factor delivery groups, 62.5 µM heparin, 0.25 mM mM AT-III peptide, 62.5 ng NT3, and 62.5 ng GDNF (DS + NT3 + GDNF) or 10 ng PDGF (DS + NT3 +PDGF) were added to each scaffold. A total of 24 scaffolds were fabricated for each group. All scaffolds were washed 5 times over a 24 hour period with 500 µl TBS per wash to remove unbound delivery system components. An individual EB containing pMNs was placed on each fibrin scaffold and covered with a 100 µl fibrin scaffold containing 10 mg/ml fibrinogen, 2.5 mM CaCl\(_2\) (Sigma), and 2 NIH units/ml thrombin (Sigma) such that the EB remained at the interface of the two fibrin layers. Fibrin scaffolds containing the single EB were
incubated at 37˚C for 1 hour. 500 ul of modified DFKNB media consisting of a 1:1 ratio of DFK5 media and neuralbasal (NB) media supplemented with 2% B27 and 5 μg/ml aprotinin was added to each well. Cells were cultured for a total of 14 days with a single media change at day 3 to NB media supplemented with 2% B27.

3.3.4 Flow Cytometry

Following the two week culture, fibrin gels were degraded with 0.25% trypsin-EDTA for 15 minutes to release cells. Cells from all 24 wells for each group were pooled in 35 mm dishes and triturated prior to quenching with an excess volume of complete media. Cells were centrifuged for 5 min at 230xg, the media was aspirated, and cells were fixed with 1% paraformaldehyde (Sigma). After fixation, the cells were permeabilized with 1% saponin (Sigma) solution for 20 min, and then blocked in 0.5% saponin solution containing 5% normal goat serum (NGS; Sigma). Cell suspensions were then incubated for 30 min in 0.5% saponin solution containing 2% NGS and one of the following primary antibodies: O4 (Millipore, 1:500), ChAT (Millipore, 1:500), NeuN (Millipore, 1:100), and GFAP (ImmunoStar; 1:100). Cells were washed with PBS and appropriate Alexa Fluor secondary antibodies (1:200; Invitrogen) diluted in 0.5% saponin with 2% NGS were applied for 30 min. Finally cells were washed with PBS and incubated with Hoechst (1:1000; Invitrogen) for 5 min.

Stained cell suspensions were analyzed using a Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For each group, 10,000 events were recorded. Subsequent analysis was performed using FloJo software (FloJo, Ashland, OR). Prior to population gating, debris was removed based on forward scatter versus side scatter and
Hoechst fluorescence versus forward scatter plots. Flow cytometry control groups, consisting of cells stained with the secondary antibody only, were used to determine quadrant population gating parameters. Flow cytometry results are presented as the percentage of cells staining positive for each marker out of the total live cell population.

### 3.3.5 Spinal Cord Injury and Scaffold Implantation

All experimental procedures on animals complied with the Guide for the Care and Use of Laboratory Animals and were performed under the supervision of the Division of Comparative Medicine at Washington University. Long-Evans female rats (250-275 g) were anesthetized using 5% isoflurane gas and 5 mg/kg xylazine. A single incision was created through the skin to expose the back muscle. Parallel incisions were created through the back muscle on each side of the vertebral processes from T5-T11. A dorsal laminectomy was performed at T8 using fine tip rongeurs to expose the spinal cord. Spinal clamps were placed in the vertebral foramina at T7 and T9 to stabilize the spinal cord. The dura mater was removed from the exposed cord at T8. Vitrectomy scissors mounted to a micromanipulator were lowered 1.2 mm into the spinal cord. A lateral incision was created across the spinal cord to from a dorsal hemisection. The injury site was covered with a piece of artificial dura and the back muscles closed using degradable sutures. Finally the skin was stapled close and the animals were treated with buprenorphine.

Two weeks following the initial incision, the injury site was re-exposed and the scar tissue removed from the spinal cord to create a cavity for scaffold implantation. Fibrin scaffolds were prepared by mixing 10 mg/ml fibrinogen, 2.5 mM CaCl₂ (Sigma),
2 NIH units/ml thrombin (Sigma), 62.5 μM heparin, 0.25 mM AT-III peptide, 125 ng NT3, and 125 ng GDNF or 20 ng PDGF per scaffold (NT3 + GDNF and NT3 + PDGF groups). For fibrin control groups (Fibrin), the following components were omitted: heparin, AT-III peptide, NT3, GDNF, PDGF. For delivery system (DS) control groups all growth factors were omitted. A 10 μl fibrin scaffold with 10 EBs containing pMNs embedded at the center of the scaffold was allowed to polymerize fully for 5 minutes prior to implantation into the injury site. Following implantation, a second 10 μl fibrin scaffold consisting of 10 mg/ml fibrinogen, 2.5 mM CaCl₂ (Sigma), and 2 NIH units/ml thrombin was allowed to polymerize in situ to hold the scaffold containing EBs within the injury site. The implantation site was then covered with artificial dura, the overlying back muscle closed using degradable sutures, and the skin stapled close.

Immediately following each surgery, animals were given cefazolin (25 mg/kg) and buprenorphine (0.04 mg/kg). Cefazolin was continued twice daily for 5 days with carprofen tablets. Bladders were manually expressed twice a day for the entire study. Following the second surgery, immune suppression was accomplished by daily injections of cyclosporine-A (10 mg/kg) to reduce immune rejection of mouse cells. Two weeks following cell transplantation, animals were euthanized by an overdose of Euthasol. Spinal cords were harvested following transcardial perfusion with 4% paraformaldehyde and post-fixed in 4% paraformaldehyde overnight. The following day, spinal cords were cryoprotected by immersion in 30% sucrose in PBS. Prior to embedding, 2 cm sections of the spinal cords with the injury site in the center were cut.
and frozen on dry ice. Cords were embedded in Tissue-Tek OCT compound and cut into 20 μm sagittal sections with a cryostat.

3.3.6 Immunocytochemistry

Immunohistochemistry was used to assess the expression of markers of differentiation for transplanted pMNs. OCT was washed from sections with PBS. Sections were permeabilized with 0.1% triton X-100 for 15 minutes and blocked with 10% bovine serum albumin and 2% normal goat serum (NGS). The following primary antibodies were applied overnight at 4˚C in 2% NGS in PBS: β-tubulin III (β-tubIII, Covance, 1:400), choline acetyltransferase (ChAT, 1:500), oligodendrocyte marker 4 (O4, 1:500), glial fibrillary acidic protein (GFAP, 1:50), neuronal nuclei (NeuN, 1:500), and nestin (1:25). Primary antibody staining was followed by 3 washes with PBS. Appropriate Alexa Fluor secondary antibodies (Invitrogen) in 2% NGS in PBS were applied for 1 hour at room temperature followed by an additional 3 washes in PBS. Cell nuclei were stained with the nuclei binding dye DAPI (1:1000). Sections were mounted using FluorMount (Invitrogen).

3.3.7 Image Analysis of Differentiation Markers

To quantify the staining of differentiation markers on transplanted EBs containing pMNs, every twentieth section from each spinal cord was analyzed. A series of 100x images spanning the lesion site were captured using a MICROfire camera attached to an Olympus IX70 inverted microscope. GFP⁺ pixels were identified in each image and the total area of GFP⁺ pixels counted per image using a custom Matlab
program (Matlab, Appendix A). Fluorescent staining for one of the six markers of differentiation (Nestin, βtubIII, GFAP, O4, NeuN, and ChAT) was assessed in each of the GFP+ pixels to determine a total area of staining using the same program. In addition, nuclei counts were performed within GFP+ areas to determine the total number of GFP+ cells in each image (Figure 3.1).

3.3.8 Statistical Analysis

Statistical significance was determined by one way analysis of variance (ANOVA) using Statistica Software (Statistica). Statistical significance was set at p < 0.05. For in vitro experiments, a Tukey’s HSD test was used to determine significance (p<0.05). Statistical significance for in vivo studies was determined by the planned comparisons post-hoc test (p<0.05).

3.4 Results

3.4.1 High Purity pMNs Survive and Differentiate in Fibrin Scaffolds

In Vitro

To determine the survival and differentiation of high-purity pMNs in fibrin scaffolds, EBs containing pMNs were selected with puromycin to remove undesired cells and encapsulated in either unmodified fibrin scaffolds (Fibrin group) or fibrin scaffolds containing the following delivery system components: heparin and AT-III peptide (DS); heparin, NT3, and PDGF (Hep + NT3 + PDGF); heparin, NT3, and GDNF (Hep + NT3 + GDNF); heparin, AT-III, NT3, and PDGF (DS + NT3 +
PDGF); or heparin, AT-III, NT3, and GDNF (DS + NT3 + GDNF). The same fibrin scaffold conditions were repeated for selection control (unselected) groups where EBs containing pMNs were not selected with puromycin. In both selected and unselected groups, axons and cells migrated from EBs (Figure 3.1). Bright GFP fluorescence was observed in encapsulated cells (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1** Cell survival and migration following a two week *in vitro* culture within fibrin scaffolds. Phase contrast (left) image of an encapsulated EB with cell migration into the fibrin gel. GFP expression (right) in cells contained within the EB and in cells migrating out of the EB. Scale bar = 100 µm.

To characterize the percentage of cells differentiating into astrocytes, oligodendrocytes, neurons, and motoneurons, flow cytometry was performed following the two week *in vitro* culture period in fibrin scaffolds. Two different combinations of growth factors were tested (NT3+GDNF and NT3+PDGF). The percentage of cells labeling with ChAT (motoneurons) was unchanged in selected and unselected groups for all of the fibrin scaffold conditions tested (Figure 3.2).
Figure 3.2 Flow cytometry analysis of pMN differentiation following a two week in vitro culture within fibrin scaffolds with (A) NT3 and GDNF growth factors and (B) NT3 and PDGF growth factors. Cell markers include O4 (oligodendrocytes), GFAP (astrocytes), NeuN (neurons), and ChAT (motoneurons). * indicates p < 0.05. Abbreviations: oligodendrocyte marker 4 (O4), glial fibrillary acidic protein (GFAP), neuronal nuclei (NeuN), choline acetyltransferase (ChAT), delivery system (DS), heparin (Hep), neurotrophin-3 (NT3), glia-derived neurotrophic factor (GDNF), platelet derived growth factor (PDGF).
A significant decrease in the number of NeuN\(^+\) neurons was observed in the Hep + NT3 + GDNF selected group and the DS + NT3 + GDNF unselected group compared to the DS selected group and the Fibrin unselected group. Overall the percentage of NeuN\(^+\) neurons and ChAT\(^+\) motoneurons was low (<10%) in all groups. A significant decrease in GFAP (astrocytes) staining was observed in the DS + NT3 + GDNF group compared to the Fibrin unselected and selected groups as well as the DS unselected group. The percentage of GFAP\(^+\) cells also decreased in the selected DS+NT3+PDGF group versus the selected fibrin group. The addition of growth factors appeared to decrease differentiation into astrocytes. Finally, significantly greater O4 (oligodendrocytes) labeling was observed in the DS + NT3 + GDNF selected condition compared to the DS + NT3 + GDNF unselected condition. In general, oligodendrocyte differentiation appeared to decrease with growth factor addition in the unselected control groups while the presence of growth factors appeared to improve oligodendrocyte differentiation in selected groups.

3.4.2 High Purity pMN\textsuperscript{s} Survive and Migrate in a Sub-acute Dorsal Hemisection Injury

The ability of high purity ES cell-derived populations to survive when transplanted in the injured spinal cord has yet to be determined. To investigate the ability for high purity pMN\textsuperscript{s} to survive when encapsulated in fibrin scaffolds we examined cell survival two weeks post-transplantation into a sub-acute dorsal hemisection SCI. Furthermore, the impact of growth factor delivery using an affinity-based delivery system on cell survival was assessed. P-Olig2 cells contained
ubiquitously expressed GFP under the β-actin promoterto facilitate visualization of transplanted cells. Four fibrin conditions were assessed: fibrin alone (Fibrin), fibrin scaffolds incorporating the affinity based delivery system (DS), fibrin scaffolds with DS, NT3, and GDNF (DS+NT3+GDNF), and fibrin scaffolds with the DS, NT3, and PDGF (DS+NT3+PDGF). Two weeks following transplantation, spinal cords were harvested and the area of GFP expression was determined. GFP+ cells were observed in the lesion cavity of all animals (Figure 3.3A). In some cases, migration of GFP+ cells into the spinal cord was observed (Figure 3.3B). No significant differences in the total GFP+ area were found between any of the fibrin conditions (Figure 3.4A). The total cell count as assessed by nuclei labeling with DAPI within GFP+ areas was similar between groups (Figure 3.4B). High purity pMNs survive and in some cases, appear to integrate into the injured spinal cord. Over-proliferation was not induced by the addition of growth factors as observed in previous studies.

3.4.3 Differentiation of Transplanted pMNs following SCI

To determine the ability for enriched pMNs to differentiate within fibrin scaffolds following transplantation into the injured cord, expression of cell-type specific markers were analyzed by immunohistochemistry two weeks post-transplantation into the sub-acute dorsal hemisection SCI. The area of 6 markers of differentiation (nestin, neural progenitors; neurons, βtubIII; astrocytes, GFAP; oligodendrocytes, O4; motoneurons, ChAT) that co-labeled with GFP was quantified for each animal (Figure 3.5). Nestin expression was low in all groups suggesting that cells had differentiated into mature cell types. The area of nestin expression was significantly decreased in the
DS+NT3+PDGF group compared to the Fibrin group. βtubIII and NeuN labeling was observed in all groups, demonstrating successful differentiation into neuronal subtypes. No significant differences in βtubIII expression were found between any groups, however, NeuN+ area was shown to be statistically greater in the DS group compared to both the DS+NT3+PDGF and the DS+NT3+GDNF group. Both astrocytes (GFAP) and oligodendrocytes (O4) were observed in all groups. No statistical differences were observed in O4+ areas between all groups. The area of GFAP expression was statistically lower in the DS+NT3+GDNF group compared to the

Figure 3.3 Immunohistochemistry of the injured spinal cord two weeks post-transplantation of pMN in the DS+HEP+NT3+PDGF group (A) Compiled overview of the dorsal hemisection injury site containing transplanted cells (GFP). Astrocytes are labeled with GFAP. (B) Magnified inset of the transplant host border. Scale bar = 500 µm. Abbreviations: glial fibrillary acidic protein (GFAP), green fluorescent protein (GFP).
Fibrin alone group. Successful differentiation into motoneurons was observed in every group as evidenced by ChAT expression. ChAT labeling followed a similar expression pattern to βtubIII. High purity pMN populations not only survive in the injured cord, but differentiate into the proper cell types including oligodendrocytes, astrocytes, and motoneurons.

### 3.5 Discussion

Differentiation of ES cells is often limited by low efficiencies. Heterogeneous transplant populations can contain undesired cell types that confound results and in some cases, lead to tumor formation. We have previously demonstrated antibiotic selection as a viable method for purification of ES cell-derived pMNs. In this study, we demonstrated that high purity pMN populations survive and differentiate into the appropriate cell types within tissue engineered fibrin scaffolds in vitro and within the injured cord. Furthermore, neuronal differentiation did not appear to be inhibited as commonly reported following transplantation into SCI environment.

Tissue-engineered fibrin scaffolds promoted subtle changes in differentiation of control unselected pMN cultures and highly enriched pMN cultures after a two week culture in vitro. The addition of growth factors appeared to increase differentiation into the oligodendrocytes fate for high purity pMNs though no statistical differences were observed. This effect was not observed in control cultures. Statistical difference was observed in between control and selected DS+NT3+GDNF groups. This may be due
Figure 3.4 Analysis of transplant cell survival within the injured spinal cord after two weeks. (A) Average total area of GFP expression in each transplant group (B) Average nuclei counts within GFP-positive areas. No significant differences were observed. Abbreviations: delivery system (DS), heparin (Hep), neurotrophin-3 (NT3), glia-derived neurotrophic factor (GDNF), platelet derived growth factor (PDGF).
to removal of confounding cell types that may respond similarly to NT3 and GDNF or that secrete factors inhibiting oligodendrocytes differentiation. In general, percentage of astrocytes appeared to decrease in both cell groups with the addition of growth factors, though statistical differences were not consistent. During the two week culture, axons from EBs containing pMN3 extended through the fibrin scaffolds. Oligodendrocytes migrated out in the scaffold away from the EB. GFP expression was maintained during the two week period and suggested survival of the encapsulated cells. Antibiotic selection improved the differentiation of ES cell-derived populations into oligodendrocytes within tissue engineered fibrin scaffolds.

High purity pMN transplants survived in the injured spinal cord and differentiate into appropriate mature cell types (neurons, oligodendrocytes and astrocytes). The presence of growth factors did not appear to influence survival or proliferation of transplanted cells contrary to results previously obtained from heterogeneous transplant populations that show greater cell numbers two weeks after transplantation. These results suggest the absence of tumor forming cell types such as undifferentiated ES cells. In many instances, cells migrated from the graft into host tissue. This may be enhanced by the lack of robust glial scarring. These results suggest that high purity pMNs differentiate into appropriate spinal cell types capable of integrating with the host spinal tissue. Indeed, transplanted cells differentiation into spinal motoneurons, oligodendrocytes and astrocytes as observed in vitro. Neuronal differentiation was observed in all grafts though greatest expression was observed in the DS group. This may be due to sequestering of growth factors expressed in the injury.
Figure 3.5 Immunohistochemical analysis of transplant cell differentiation within the injured spinal cord after two weeks. (A) Average area of nestin (neural progenitors) expression. (B) Average area of βtubIII (immature neurons) expression. (C) Average area of NeuN (mature neurons) expression. (D) Average area of ChAT (motoneurons) expression. (E) Average area of GFAP (astrocytes) expression. (F) Average area of O4 (oligodendrocytes) expression. * indicates p < 0.05. Abbreviations: β-tubulin class III (βtubIII), oligodendrocyte marker 4 (O4), glial fibrillary acidic protein (GFAP), neuronal nuclei (NeuN), choline acetyltransferase (ChAT), delivery system (DS), heparin (Hep), neurotrophin-3 (NT3), glia-derived neurotrophic factor (GDNF), platelet derived growth factor (PDGF).
site by unoccupied heparin sites present in the scaffold. Similar to in vitro studies, the NT3 + GDNF growth factor combination reduced astrocyte differentiation. These results suggest that in vitro studies may be able to approximate in vivo results, at least in the case of high purity pMNs. Transgenic enrichment of pMNs appears to generate a promising cell transplant populations for treatment after SCI.

The development of high purity ES cell-derived transplant populations can benefit cell replacement therapies for SCI. This study demonstrates the feasibility of high purity pMN populations for repair of the injured cord. Future studies to elucidate the effect of this cell population on functional recovery can further its therapeutic application. Antibiotic selection appears to be a safe and effective mechanism for improving ES cell-derived cell transplant populations.
Chapter 4

Generation of High Purity Cholinergic Motoneurons by Hb9-Enhancer Driven Antibiotic Resistance in Genetically Engineered Mouse Embryonic Stem Cells

4.1 Abstract

Many techniques have been developed to isolate motoneurons from heterogeneous cell populations; however, their utility for purification of embryonic stem (ES) cell-derived motoneurons has not been fully developed. We engineered a transgenic-ES cell line that allows antibiotic selection to purify motoneurons following differentiation of mouse ES cells. Highly conserved enhancer elements for the motoneuron transcription factor Hb9 were used to drive puromycin N-acetyltransferase (PAC) expression in ES cell-derived motoneurons. Following puromycin selection, high purity motoneuron cultures were obtained. Purity was maintained during maturation to produce consistent, uniform populations of cholinergic ES cell-derived motoneurons. Functional properties of purified motoneurons were examined by acetyl cholinesterase (AChE) activity and electrophysiology. Puromycin-selected motoneurons exhibited appropriate voltage-gated and ligand-gated ion channel activity with the ability to fire multiple action potentials. Selected cultures were post-mitotic and did not contain astrocytes or other proliferating glia frequently observed in ES cell-derived motoneuron cultures. Antibiotic selection, therefore, can provide an inexpensive alternative to current methods for isolating motoneurons at high purity that does not require specialized laboratory equipment.
4.2 Introduction

The ability to culture neurons in vitro has many advantages including applications in toxicology screening, developmental studies, and cell replacement strategies. ES cells hold great potential as an expandable cell source that can be differentiated into specific neuronal sub-types by recapitulating developmental signals. The signaling events necessary for differentiation of ES cells into midbrain dopaminergic neurons (Lee et al., 2000), cortical pyramidal neurons (Gaspard et al., 2009), cerebellar neurons (Salero and Hatten, 2007), dorsal interneurons (Murashov et al., 2005), and spinal motoneurons (Wichterle et al., 2002) have been previously described. ES cell-derived neurons have been shown to maintain neuronal sub-type specific properties and have the potential to integrate when transplanted into appropriate regions of the central nervous system (Kim et al., 2002, Wichterle et al., 2009, Espuny-Camacho et al., 2013). Furthermore, transplanted mouse ES cell-derived motoneurons have been shown to restore partial motor function following selective ablation of host spinal motoneurons in rats (Deshpande et al., 2006).

The directed differentiation of ES cells into spinal motoneurons can be achieved by exposure of embryoid bodies (EBs) to retinoic acid (RA) and sonic hedgehog (Shh) (Wichterle and Peljto, 2008). RA serves as a caudalizing signal to generate spinal progenitor cells, while Shh acts as a ventralizing agent to induce differentiation into progenitor motoneurons (pMNs) expressing the basic helix-loop-helix transcription factor Olig2. Spinal motoneurons differentiate from pMNs by expressing the homeobox domain transcription factor Hb9 and down-regulating Olig2 (Arber et al., 1999). In addition to motoneurons, pMNs also give rise to oligodendrocytes and
astrocytes in vitro (Xian and Gottlieb, 2004). The efficiency of differentiation into pMN

pMN can be enhanced by use of small molecule agonists of the Shh pathway, resulting in up to up to 50% of the total cell population expressing Hb9 (Li et al., 2008). Post-mitotic motoneurons, however, are diluted as a percentage of the total cell population by the continuing proliferation of glia during extended culture. High purity mature motoneuron cultures may be desired to evaluate neuron-glial interactions and to maintain independent cultures, as well as for cell transplantation.

Several methods have been developed to purify motoneurons from mixed cell cultures. Isolation of MN from mouse (Gingras et al., 2007), rat (Schnaar and Schaffner, 1981), and chick (Schnaar and Schaffner, 1981) fetal spinal cord has been demonstrated using density gradient centrifugation to separate large motoneurons based on cell density. While initially successful, this technique cannot guarantee removal of glia. When applied to human and monkey ES cell-derived motoneurons, gradient centrifugation provided partial enrichment of motoneurons (Wada et al., 2009). Purity following extended culture of enriched human ES cell-derived motoneurons, however, was not assessed. Alternatively, the Hb9 promoter has been used to drive enhanced green fluorescent protein (eGFP) expression for visual identification and fluorescence activated cell sorting (FACS) of ES cell-derived motoneurons (Singh Roy et al., 2005). FACS, however, is time intensive and requires a central facility with expensive equipment, and it also has the potential to contaminate cell cultures. Other methods have been utilized to purify motoneurons, including panning for the low affinity nerve growth factor receptor (p75), but these approaches may limited by specificity and technical difficulty (Camu and Henderson, 1992).
The development of a low-cost, reproducible technique for producing high purity ES cell-derived motoneurons can provide a powerful tool to enable studies of neurodegenerative diseases and development. In this study, we obtained high purity cultures by antibiotic selection of ES cell-derived motoneurons. Mouse ES cells were transfected with a selection vector containing two highly conserved enhancers of the Hb9 gene driving the puromycin resistance enzyme, PAC. Following differentiation of ES cells into motoneurons, addition of puromycin to the culture media killed cells not expressing Hb9. Motoneuron purity was assessed immediately following puromycin selection and was maintained through maturation cultures. High-purity motoneurons matured into functional cholinergic neurons as determined by an AChE activity assay and electrophysiology. This technique provides an inexpensive and scalable method for obtaining high purity mature motoneuron cultures.

4.3 Materials and Methods

4.3.1 Embryonic Stem Cell Culture

Mouse ES cells were cultured on gelatin-coated T-25 flasks in complete media consisting of Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) containing 10% newborn calf serum (Life Technologies), 10% fetal bovine serum (Life Technologies), 10 μM thymidine (Sigma, St. Louis, MO), and 30 μM of each of the following nucleosides (Sigma): adenosine, cytosine, guanosine, and uridine. For routine culture, ES cells were passaged every two days. Briefly, ES cells were washed with DMEM containing 25 mM HEPES (Life Technologies) and
dissociated with 0.25% trypsin-EDTA (Life Technologies) for 5 min. Trypsin was quenched with fresh complete media and cells were transferred to a new gelatin-coated flask at a 1:5 ratio in fresh complete media containing 1000 U/mL leukemia inhibitory factor (LIF; Millipore, Bellerica, MA) and 100 μM β-mercaptoethanol (BME; Life Technologies).

### 4.3.2 Hb9-Puro Selection Vector

Hb9 enhancers A and B followed by the minimal β-globin promoter were PCR amplified from the CSH2IV vector (kind gift from Patrick Blanner). The resulting PCR product was cut and ligated into a vector containing the puromycin resistance cassette (kind gift from David Gottlieb). The resistance cassette also contained the gene for a neomycin resistance enzyme, neomycin phosphotransferase, driven by the ubiquitously expressed phosphoglycerate kinase (PGK) promoter for identification of integration events. The PGK promoter and the neomycin phosphotransferase gene were flanked by loxP sites to facilitate removal following integration of the Hb9-Puro vector. Ligated vector was amplified in DH5α E. Coli and isolated using a mini-prep kit (Qiagen, Hilden, DE).

### 4.3.3 Electroporation and Cre Excision

RW4 ES cells were used to make a transgenic ES cell line. Approximately 10 x 10⁶ ES cells were resuspended in electroporation buffer with 10 μg Sal1 linearized Hb9-Puro selection vector. Cells were electroporated at 0.23 kV and 960 μF in a 0.4 cm cuvette (Bio-Rad, Hercules, CA) and plated on 100 mm gelatin-coated petri dishes in
complete media with 1000 U/mL LIF, 100 μM BME, and 40 ug/mL geneticin (Life Technologies). Media was replaced every 2 days. After 9 days, single colonies were picked and plated in individual wells of a gelatin-coated 48 well plate containing complete media with 1000 U/mL LIF and 100 μM BME. Clones were screened for puromycin resistance at the ES cell state and resistant clones were discarded. The pTurboCre plasmid (kind gift from David Gottlieb) was used to remove the PGK promoter and neomycin phosphotransferase gene from transgenic ES cells. Approximately 3 ng plasmid was incubated in Opti-MEM media (Life Technologies) and Lipofectamine 2000 (Life Technologies). Half of the culture media was replaced with Opti-MEM media containing pTurboCre plasmid and Lipofectamine 2000 for 4 hours. Media was then replaced with complete media containing LIF and BME and cells were allowed to recover overnight. The following day, ES cell cultures were dissociated and plated at 6 x 10^3 cells/cm² in individual wells of a gelatin-coated 6 well plate in complete media with 1000 U/mL LIF, 100 μM BME, and 40 ug/mL geneticin. After 9 days, single colonies were picked and plated in individual wells of a gelatin-coated 48 well plate containing complete media with LIF and BME. Clones were screened for sensitivity to neomycin by exposure to 40 µg/ml geneticin for 5 days, and those that were not sensitive were discarded.

4.3.4 Motoneuron Differentiation

Transgenic ES cells were differentiated into motoneurons using a 2'/4' RA and smoothened agonist (SAG, Millipore) induction protocol as previously described (McCreedy et al., 2012). Approximately 1 x 10^6 ES cells were cultured in suspension on
agar-coated 100 mm petri dishes in modified DFK5 media consisting of DMEM/F12 base media (Life Technologies) containing 5% knockout serum replacement (Life Technologies), 1x insulin transferrin selenium (ITS; Life Technologies), 50 μM nonessential amino acids (Life Technologies), 100 μM β-mercaptoethanol, 5 μM thymidine, and 15 μM of the following nucleosides: adenosine, cytosine, guanosine, and uridine. During this process, ES cells aggregate into multi-cellular EBs. After the first two days (2), the EBs were moved to a 15 mL conical and allowed to settle for 5 min. The media was aspirated and replaced with 10 mL fresh DFK5 containing 2 μM RA and 600 nM SAG. In differentiated control cultures, the Shh antagonist cyclopamine (Cyc, 1 μM; Sigma) was used instead of SAG. EBs were then cultured for an additional 4 days (4+), and media was replaced every 2 days.

4.3.5 Motoneuron Selection

Transgenic ES cells differentiated using the 2+/4+ RA and SAG induction were dissociated in 0.25% Trypsin-EDTA for 15 minutes and quenched with complete media. Dissociated cells were counted and centrifuged at 240 x g for 5 minutes. Cells were resuspended in DFK5 media containing 5 ng/mL glial-derived neurotrophic factor (GDNF; Peprotech, Rocky Hill, NJ), 5 ng/mL brain derived neurotrophic factor (BDNF; Peprotech, Rocky Hill, NJ), 5 ng/mL neurotrophin-3 (NT-3; Peprotech, Rocky Hill, NJ), 4 μg/mL puromycin in water (Sigma) and plated at 6 x 10^4 cells/cm² in individual wells of a laminin-coated 6 well plate for 24 hours. In parallel control cultures not receiving puromycin, cells were resuspended in DFK5 media with the growth factor cocktail and plated at 3 x 10^4 cells/cm² in individual wells of a laminin-
coated 6 well plate for 24 hours. Following selection, cells were fixed for immunofluorescence or media was replaced with modified DFKNB media consisting of DFK5 and Neural basal (NB) media (Life Technologies) mixed at a 1:1 ratio and supplemented with B27, 5 ng/mL GDNF, 5 ng/mL BDNF, and 5 ng/mL NT-3. Cells were cultured in DFKNB media for up to 8 additional days.

4.3.6 Polymerase Chain Reaction

Cell lysates were collected using an RNeasy kit (Qiagen) from ES cells, ES cells differentiated with the 2/4+ RA and SAG or the 2/4+ RA and Cyc induction protocol. 50 ng mRNA from each sample was amplified using a One-Step PCR Kit (Qiagen). Primers for Hb9 were as follows: 5'-GGCCATAGGATGGGATTGTA-3'(forward), 5'-CGGCGCTTCCTACTCATC-3'(reverse).

4.3.7 Cell Viability Assay

To determine cell viability after puromycin selection, ES cells were differentiated using the 2/4+ RA and SAG or the control 2/4+ RA and Cyc induction protocol. Differentiated cells and undifferentiated ES cells were dissociated and plated in individual wells of laminin-coated 6 well plate in DFK5 media with 4 μg/mL puromycin at a density of 6 x 10⁴ cells/cm² and 4 x 10⁴ cells/cm², respectively. Cells were selected with puromycin for 24 hours. Control cultures not receiving puromycin were run in parallel. Following puromycin selection, media was replaced with fresh DFK5 containing calcein-AM (Life Technologies) for 30 minutes. Fluorescent images were
captured using a MICROfire camera attached to an Olympus IX70 inverted microscope.

### 4.3.8 Transcription Factor Analysis

Due to the short temporal expression of the Olig2 and Hb9 transcription factors, ES cells were differentiated using a modified 2/4^+ RA and SAG exposure protocol, where EBs were dissociated at 2/3^+ and plated in individual wells of a laminin-coated 6-well plates in fresh DFK5 media containing 2 μm RA, 600 nM SAG, and 2 μg/mL puromycin for 20 hours prior to fixation for immunofluorescence. Control cultures without puromycin for the 20 hour selection period were run in parallel. Cultures were fixed following selection and analyzed by immunofluorescence (see Immunofluorescence and Cell Counting).

### 4.3.9 Immunofluorescence and Cell Counting

Cells were fixed in 4% (w/v) paraformaldehyde (Sigma) in phosphate buffered saline (PBS) for 15 minutes at room temperature. Fixed cultures were washed once with PBS, and the cells were permeabilized with 0.1% triton-X in PBS for 15 minutes. Cultures were then blocked for 1 hour at 4°C with 5% normal goat serum (NGS; Sigma) in PBS. Primary antibodies were added to blocked cultures overnight at 4°C at the following dilutions in 2% NGS in PBS: neurofilament (NF, 1:50, Iowa Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), Olig2 (1:400, Millipore), MNR2/Hb9 (1:20, DSHB), β-tubulin III (β-TubIII, 1:400, Millipore), choline acetyltransferase (ChAT, Millipore), ChAT (ChAT4B, DSHB), or glial fibrillary
acidic protein (GFAP, 1:100, ImmunoStar, Hudson, WI). Primary antibodies were labeled with the appropriate AlexaFluor conjugated goat antibodies (Life Technologies) at a 1:200 dilution in 2% NGS in PBS for 4 hours. All wells were counterstained with the Hoechst (1:1000; Life Technologies) to label cell nuclei. Following immunofluorescence, phase contrast and fluorescent images were captured. Live cells were identified by phase contrast and Hoechst staining. Cells with apparent processes and round nuclei were included in the cell counting experiments. Cells with fragmented nuclei demonstrating evidence of cell death were not included in total cell counts. Cell counting was performed using ImageJ software.

4.3.10 AChE Staining

A modified Karnovsky stain was used to assess AChE activity in motoneurons. Cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 30 minutes. The wells were washed with water and incubated in AChE staining solution consisting of 65mM phosphate buffer (pH = 6.0; Sigma), 10 mg/mL gelatin (Sigma), 5 mM sodium citrate (Sigma), 3 mM copper sulfate (Sigma), 0.5 mM potassium ferricyanide (Sigma), 0.2 mM ethropropazine (Sigma) and 4 mM acetylthiocholine (Sigma) for 7 days. Wells were rinse 5 times with water. Water was removed and Prolong Gold mounting solution containing DAPI (Life Technologies) was added to each well. A coverslip was carefully placed over the well and mounting solution was allowed to harden overnight at room temperature. Brightfield images and fluorescent images were taken the following day.
4.3.11 Electrophysiology

Cultures were bath perfused with Tyrode’s solution (in mM): 150 NaCl, 4 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH. Whole-cell electrodes pulled from borosilicate glass capillaries had an open tip resistance of 2 to 5 MOhm when filled with one of the following internal solutions (in mM): (1) 140 K-glucuronate, 10 NaCl, 5 MgCl$_2$, 0.2 EGTA, 5 Na-ATP, 1 Na-GTP, 10 HEPES, pH adjusted to 7.4 with KOH; (2) 140 Cs-glucuronate, 5 CsCl, 5 MgCl$_2$, 10 EGTA, 5 Na-ATP, 1 Na-GTP, 10 HEPES, pH adjusted to 7.4 with CsOH; or, (3) 120 tetraethylammonium (TEA) chloride, 5 MgCl$_2$, 5 Na-ATP, 1 Na-GTP, 10 EGTA, 10 HEPES, pH adjusted to 7.4 with TEA-hydroxide. Current and voltage were recorded with an Axopatch 200A amplifier, filtered at 1 kHz, digitized at 10 kHz and analyzed off-line with Clampfit software (pClamp 9.2). Voltage-gated channel antagonists tetrodotoxin (TTX) and 4-aminopyridine (4-AP) were dissolved in Tyrode’s solution and delivered under gravity flow from a local perfusion pipette (Kim et al., 2009). Ligand-gated channel agonists were dissolved in 160 NaCl, 10 HEPES, 2 CaCl$_2$ and also applied by local perfusion.

4.3.12 Statistical Analysis

Unless otherwise noted all measured parameters are reported as mean +/- SEM. Statistical significance was determined by Scheffe’s post hoc test for analysis of variance (ANOVA) using Statistica software (version 5.5; StatSoft, Tulsa, OK). Significance was assigned for P values less than 0.05.
4.4 Results

4.4.1 Hb9 Enhancers Drive Puromycin Resistance Following Differentiation of Embryonic Stem Cells into Motoneurons

Differentiation of ES cells using RA and Shh gives rise to multiple cell types (Figure 4.1). To isolate motoneurons from the diverse cell population, we developed a new selection vector (Hb9-Puro) containing previously identified enhancer elements of the motoneuron transcription factor, Hb9, driving the puromycin resistance enzyme, PAC (Figure 4.2A) (Nakano et al., 2005). The Hb9-Puro selection vector was electroporated into ES cells, and the resulting cultures were screened for neomycin resistance to ensure genomic incorporation of the vector. Transgenic ES cells were then treated with cre recombinase to remove the neomycin selection cassette that is flanked by loxP sites. The transgenic ES cell cultures were aggregated for two days (2) to form EBs as part of a 2/4+ induction protocol. EBs were exposed to RA and SAG, for four days (4+) to induce differentiation into motoneurons. Undifferentiated ES cells and ES cells differentiated with RA and the Shh antagonist, Cyc were used as controls. Cells from each group were lysed and presence of Hb9 mRNA was assessed by RT-PCR. In cultures receiving RA and SAG, Hb9 mRNA was detected by PCR (Figure 4.2B). No Hb9 mRNA was observed in either control group.

To determine if Hb9 expression led to expression of PAC, cultures differentiated with RA and SAG were dissociated following the 2/4+ induction protocol and plated on laminin-coated wells with puromycin (4 μg/mL) in the media for 24 hours. Parallel cultures not receiving puromycin, as well as undifferentiated ES cells and ES cells differentiated with RA and Cyc, were used as controls. In the absence
of puromycin, all cultures contained viable cells as evidenced by calcien AM staining (Figure 4.2C). With puromycin, undifferentiated ES cells and ES cells differentiated with RA and Cyc did not survive. Viable cells, however, were present following puromycin selection for ES cells differentiated with RA and SAG. Surviving cells displayed phase bright cell bodies and long neurites, morphology consistent with neuronal differentiation.

4.4.2 Surviving Cells Label for Immature Motoneuron Markers

Immediately Following Puromycin Selection

To determine if Hb9 enhancer-driven puromycin resistance was specific to motoneurons, we investigated the expression of Olig2 and Hb9, two transcription factors associated with motoneuron development and differentiation. Due to the short
Figure 4.2 Hb9-Puro selection vector and puromycin resistance. (A) Schematic showing location of Hb9 enhancers A and B upstream of Hb9 gene. Enhancers A and B were used in conjunction with the minimal β-globin promoter (β) to drive PAC expression. Neomycin phosphotransferase expression was driven by the ubiquitously expressed PGK promoter for identification of cells with incorporated vector. Cre recombinase was used to remove the PGK promoter and neomycin phosphotransferase gene following incorporation into ES cells. (B) Hb9 mRNA expression in ES cells (ES), ES cells differentiated with RA and Cyc (Cyc), and ES cells differentiated with RA and SAG (SAG). (C) Live cell assay in ES cells, ES cells differentiated with RA and Cyc (Cyc), and ES cells differentiated with RA and SAG (SAG) following puromycin selection. Control cultures were run in parallel without puromycin.
temporal expression of Olig2 and Hb9, a modified 2/4+ induction and selection protocol was used. ES cells were aggregated for two days into EBs and exposed to RA and SAG for three days (2/3+). EBs were then dissociated and plated on laminin-coated plates with puromycin, RA, and SAG for 20 hours (Figure 4.3A). In parallel cultures not receiving puromycin,, 32.3 ± 1.7% of cells stained positive for the pMN transcription factor Olig2 and 46.3 ± 1.0% of cells stained positive for motoneuron transcription factor Hb9 (Figure 4.3B-C). When puromycin was added during the 20 hr selection period, no cells labeled for Olig2 (n=3, p<0.001) while 98.3 ± 0.10% of cells were positive for Hb9 (n=3, p<0.001). Remaining viable cells had a consistent neuronal morphology.

To increase the number of surviving cells for analysis of the neuron-specific marker β-tubulin class III (β-tubIII), ES cells were aggregated for 2 days then exposed to RA and SAG for 4 days (2/4+), at which time Hb9 expression peaks. EBs were then dissociated and plated on laminin-coated wells for 24 hours with puromycin (Figure 4.4A). In parallel control cultures without puromycin, many cells were observed with glial morphology and did not express β-tubIII (Figure 4.4B). Approximately 59.7 ± 2.79% of cells stained positive for β-tubIII at the end of the 24 hour period in control cultures without puromycin selection. In cultures receiving puromycin for 24 hours, cells with glial morphology were not observed. The percentage of cells expressing β-tubIII significantly increased to 98.9 ± 0.20% following puromycin selection (n=3, p<0.001, Figure 4.4C). The remaining viable cells also predominantly express the neuronal marker MAP-2 (Figure 4.5).
Figure 4.3 Puromycin selection of transgenic ES cells. (A) Schematic of modified 2/4+ induction protocol including the 20-hour puromycin selection. Puromycin was added for 20 hours between day 5 and day 6. (B) Immunofluorescence analysis of Olig2 and Hb9 transcription factors following puromycin selection in culture not receiving puromycin (control) and selected with 4 µg/mL puromycin (puromycin). (C) Counting analysis of control and puromycin selected cultures. * indicates p < 0.001 for each marker compared to control group. Scale bars = 100 µM.
4.4.3 High Purity Motoneurons Achieve Functional Maturation

To determine if immature motoneurons could achieve functional maturation following puromycin selection, ES cells were differentiated using the 2/4+ RA and Shh induction protocol and selected with puromycin for 24 hours on laminin-coated wells. Cultures without puromycin were run in parallel. Following selection, cultures were switched to DFKNB media without puromycin for 5 days prior to fixation (Figure 4.6A). In selected cultures, cells displayed large neuronal cell bodies and long branching neurites, morphology typical of mature motoneurons. When labeled for the mature neuronal marker neurofilament (NF), 99.4 ± 0.61% of cells were NF-positive in selected cultures while only 5.42 ± 1.24% of cells expressed NF in control cultures not receiving puromycin (n=3, p<0.001, Figure 4.6B). Large NF+ neurites were observed extending between cell bodies of neurons in both cultures (Figure 4.6C).

Mature motoneurons contain AChE and choline acetyl-transferase (ChAT) enzymatic activity. Poor staining of ChAT antibodies by immunofluorescence in vitro has been previously reported (Bohl et al., 2008). Karnovsky staining has been utilized to differentially label motor nerves from sensory nerves via AChE activity (Kanaya et al., 1991). Similar assays have been used to label motoneurons in culture where antibodies for ChAT were inconclusive. We employed a modified version of the Karnovsky stain to assess AChE activity in fixed cultures (Gruber and Zenker, 1973, Kanaya et al., 1991, Bohl et al., 2008). In this process the enzymatic activity of AChE facilitates a chemical reaction resulting in Hatchett’s Brown precipitate. In control cultures, precipitate was observed in cells with neuronal morphology (Figure 4.6c). Labeled cells accounted for 30.6 ± 1.67% of cells in the absence of puromycin. Significantly more cells, 99.3 ±
Figure 4.4 Puromycin selection of transgenic ES cells. (A) Schematic of 2/4+ induction protocol followed by a 24-hour puromycin selection. Puromycin was added for 24 hours between day 6 and day 7. (B) Immunofluorescence analysis of β-tubulin class III (β-tubIII) following puromycin selection. (C) Counting analysis of control and puromycin selected cultures. * indicates p < 0.001 for each marker compared to control group. Scale bars = 100 µM.
0.43% (n=3, p<0.001), contained precipitate in selected cultures and all cells displayed neuronal morphology (Figure 4.6B-C). Cultures were also stained with two separate ChAT antibodies (Figure 4.7). Bright fluorescence was observed in control and selected cultures. In control cultures, dim non-specific staining was observed in cells with glial morphology preventing accurate assessment of cell purity. Non-specific staining was not observed in selected cultures.

4.4.4 Selected Cultures Are Post-Mitotic and Maintain Purity after Extended Maturation

Neural differentiation of ES cells results in many glial sub-types that rapidly proliferate and reduce neuronal purity. Astrocytes are one of the first mature glial cells
to differentiate from glial progenitors. To evaluate the presence of astrocytes, cultures were allowed to mature for 7 days following puromycin selection (Figure 4.8A). Cultures were then fixed and labeled for glial fibrillary acidic protein (GFAP). In control cultures not receiving puromycin, GFAP+ astrocytes were frequently observed (Figure 4.8B). No GFAP labeling, however, was observed in any cultures receiving puromycin for 24 hours. Cells in selected cultures maintained neuronal morphology with large cell bodies and branched neurites.

Motoneurons are post-mitotic and will not proliferate like their glial counterparts. To assess proliferation, ES cells were differentiated using the 2/4+ RA and SAG protocol and plated on laminin-coated-wells for 24 hours with puromycin in the culture media. Control cultures without puromycin were run in parallel. The total number of cell nuclei was assessed immediately following selection, at 3 days, and at 5 days post-selection. Control cultures contained approximately $2.96 \times 10^3$ cells/cm$^2$, while selected cultures contained approximately $9.3 \times 10^3$ cells/cm$^2$ post-selection. The number of cells in control cultures increased to $8.27 \times 10^3$ cells/cm$^2$ and $1.478 \times 10^4$ cells/cm$^2$ at 3 and 5 days after the selection period, respectively. Normalized to the first day following selection, there was approximately a 2.8-fold and 5-fold increase in cells for control cultures not receiving puromycin (Figure 4.8C). A decrease in cell number was observed in selected cultures to $6.4 \times 10^3$ cells/cm$^2$ (0.68 fold change) and $5.8 \times 10^3$ cells/cm$^2$ (0.62 fold change) at 3 and 5 days post-selection suggesting that proliferating cells were absent. The fold changes in control and selected cultures were significantly different at 3 and 5 days post selection ($n=3$, $p<0.05$).
4.4.5 Selected Cultures Maintain Mature Electrophysiological Properties

To determine whether our puromycin selection process had any detrimental effects on the ability of ES cell-derived motoneurons to undergo functional differentiation, we performed whole-cell current- and voltage-clamp recordings between 0 and 7 days.

**Figure 4.6** Maturation of puromycin selected motoneurons. (A) Schematic of 2:4+ induction protocol followed by a 24-hour puromycin selection and 5 day extended culture. Puromycin was added for 24 hours between day 6 and day 7. Media was replaced with DFKNB media immediately following selection and cultures were continued for an additional 5 days (B) Counting analysis of control and puromycin selected cultures for neurofilament (NF) marker and acetylcholinesterase (AChE) assay. (C) Immunofluorescence analysis of NF and AChE following 5 day extended culture. * indicates $p < 0.001$ for each marker compared to control group. Scale bars = 100 µM.
after puromycin selection (d0 to d7). When stimulated by depolarizing current injection, cells recorded under current clamp at d2 to d7 fired action potentials (Figure 6A) with a mean threshold of $-34.1 \pm 1.5$ mV ($n=19$). More than half of the cells maintained a negative resting membrane potential ($<-50$ mV) without the need for DC hyperpolarization. The average resting membrane potential ($V_{rest}$) in these cells was $-60.4 \pm 1.2$ mV ($n=10$). For long duration current pulses (800 msec) roughly one third of the
cells only fired single action potentials, another third fired multiple spikes, but with low
frequency (10-15 Hz) and substantial adaptation in spike amplitude, while the remaining
cells fired repeatedly at 20-25 Hz and with much less spike amplitude adaptation (Figure
4.9A, Figure 4.10).

Under voltage-clamp, steps from a holding potential of -80 mV to more
depolarized test potentials evoked fast transient inward currents (Figure 4.9B) that were
blocked by the sodium channel antagonist tetrodotoxin (TTX); and, more slowly
activating, and partially inactivating, outward currents (Figure 4.9C) that were largely
eliminated when potassium in the internal solution was substituted with cesium (Cs) or
tetraethylammonium (TEA). In addition, the transient component of outward current
was selectively inhibited by holding at -40 mV or by extracellular exposure to 5 mM 4-
aminopyridine (4-AP) (Figure 4.9C). Lower doses of 4-AP (500 µM), which selectively
block transient outward current in some cell types (Bean, 2007), had minimal effect on
currents recorded in our puromycin-selected motoneurons (n=6). In addition to
voltage-gated sodium and potassium currents, the selected motoneurons also displayed
cadmium-sensitive currents mediated by voltage-gated calcium channels (Figure 4.9D),
which were recorded using 5 or 25 mM barium as the charge carrier and with both
internal and external monovalent cations largely replaced by TEA. Table 1 summarizes
parameters in the puromycin-selected motoneurons for activation and steady-state
inactivation of voltage-gated sodium and transient potassium currents, as well as
activation parameters for sustained potassium and calcium currents (Figure 4.11).
To test for expression of functional neurotransmitter receptors we exposed cells to selective agonists for AMPA/kainate, NMDA, glycine, and GABA receptors, all of which activated inward current while holding cells at a fixed potential of -80 mV (Figure 4.12A). To evaluate the permeation properties of channels that underlie these responses, we applied the agonists while ramping the membrane potential from -100 to +100 mV (Figure 4.12B). As expected for inhibitory chloride-selective channels the currents evoked by glycine and GABA reversed polarity near -50 mV, which is close to the

**Figure 4.8** Glial differentiation and proliferation following puromycin selection. (A) Schematic of 2-/4+ induction protocol followed by a 24-hour puromycin selection and 7 day extended culture. Puromycin was added for 24 hours between day 6 and day 7. Media was replaced with maturation media immediately following selection and cultures were continued for an additional 7 days (B) Immunofluorescence analysis of glial fibrillary acidic protein (GFAP, astrocytes) expression in culture 7 days post-selection. (C) Total nuclei counts immediately post-selection (Day 0) continuing 3 and 5 days post-selection. Total nuclei number was normalized to Day 0. * indicates p < 0.05 for selected group compared to control group. Scale bars = 100 µM.
calculated equilibrium potential for chloride during these recordings (-54 mV). In contrast, the currents evoked by excitatory glutamate receptor agonists kainate and NMDA reversed polarity near 0 mV, consistent with activation of non-selective cation channels (Traynelis et al., 2010). Figure 4.12C plots the mean amplitude of agonist-activated currents recorded 0, 2, 4 and 6d after puromycin selection showing that responses increased as differentiation proceeded. Over this same time period whole-cell capacitance, which is proportional to surface area, increased from 22.6 ± 1.5 pF at d0 (n=11) to a plateau of 54.1 ± 2.7 pF between d5 and d8 (n=52). Taken together, our physiological data are largely in agreement with previous work on motoneurons derived from in vitro differentiation of mouse (Miles et al., 2004) and human (Takazawa et al., 2012) ES cells, and with studies of primary motoneurons in dissociated culture (Carrascal et al., 2005).

4.5 Discussion

ES cells provide an important tool that allows the study of a variety of cell types in culture that may be difficult to isolate from primary tissue. Obtaining high purity ES cell-derived populations, however, can be difficult due to the heterogeneity in cultures obtained following current differentiation protocols. The MYH6 promoter has been previously used to drive puromycin resistance in ES cell-derived cardiomyocytes leading to significant enrichment (91.5 ± 4.3% purity) following selection (Anderson et al., 2007). We have recently used homologous recombination at the Olig2 locus to confer puromycin resistance and isolate pMNs from differentiated ES cell cultures (McCreedy et al., 2012). While promising, these studies have failed to produce near pure cultures
Figure 4.9 Action potentials and voltage-gated whole-cell currents. (A) Sub- and suprathreshold voltage responses recorded under current clamp with 50 msec square pulse current injections (30 and 34 pA); examples of action potential firing patterns in 3 different cells stimulated with 800 msec square pulse current injections (K-glucuronate internal solution, d7 after selection). (B) Voltage-gated sodium channel currents evoked by steps from -60 to +12 mV from a holding potential of -80 mV; (below) Current-voltage relation for peak inward currents. (TEA-chloride internal solution, d5 after selection). (C) Transient and sustained voltage-gated potassium channel currents evoked by steps from -100 to +60 mV from holding potentials of -80 and/or -40 mV. (left) Transient currents revealed by subtracting non-inactivating currents evoked while holding at -40 mV from the combined currents evoked...
 (>99%) of a single cell type. The identification of two unique enhancers of the motoneurons associated gene Hb9, or MNX1 in the human genome, has allowed for specific labeling of motoneurons within the mouse spinal cord (Nakano et al., 2005). Furthermore, a 3.6 kb region including these enhancers has been used to drive GFP expression in ES cell-derived motoneurons for FACS (Singh Roy et al., 2005). To determine if these enhancers could drive puromycin resistance in motoneurons, we created a selection vector with the Hb9 enhancers driving expression of the puromycin resistance enzyme, PAC.

Transgenic mouse ES cells, generated by electroporation of a linearized Hb9-Puro selection vector, maintained normal motoneuron differentiation with Hb9 mRNA only detected following the 2/4+ RA and Shh induction protocol. The Shh antagonist, Cyc inhibited any detectable expression of Hb9 by PCR. Viable cells were only observed agonist, SAG. These data indicate that expression of PAC correlates with Hb9 expression as expected. Furthermore, the two Hb9 enhancers in tandem were sufficient, along with the minimal β-globin promoter, to provide puromycin resistance.
The highly conserved nature of these enhancers suggests that the Hb9-Puro selection vector may be appropriate for ES cells from other species including human ES cells.

In motoneurons development, Olig2 expression precedes the expression of Hb9. Following commitment to the motoneurons fate, Hb9 is up-regulated as Olig2 is down-regulated. In selected cultures, no Olig2 expression was observed. The absence of Olig2+ cells demonstrates the appropriate temporal regulation of the Hb9-Puro selection vector. Selected cultures stained positive for other appropriate markers for immature neurons including β-tubulin class III (β-tubIII) and MAP-2. These results demonstrate that expression of PAC by the Hb9-Puro selection vector is temporally and spatially restricted to cells committed to the motoneuron fate and can be used to generate high purity (>99%) cultures.

In studies focused on isolating motoneurons from heterogeneous cell populations, the extended culture of motoneurons beyond 2 days while maintaining purity has yet to be demonstrated (Gingras et al., 2007, Wada et al., 2009). Furthermore, the maturation of motoneurons in the absence of astrocyte feeder layers is limited. In the current study, greater than 99% of the cells in selected cultures stained positive for the neuronal marker NF at the end of the extended cultures (up to 5 days). Selected motoneurons were able to mature while neuronal purity was maintained at 5 days post-selection. High purity cultures also labeled with the AChE assay, demonstrating appropriate enzymatic activity. AChE is found in any neuronal sub-type capable of receiving cholinergic input and is not specific to motoneurons. ChAT provides a more specific marker for motoneurons. While ChAT immunolabeling was observed with two different antibodies, counting was not possible due to non-specific
Figure 4.10 Action potential properties in selected motoneurons. (A) Histogram of firing pattern as a function of days after selection. Cells spiking was classified as Single, Adapting or Multiple for 800 msec square pulse stimulation as illustrated in Figure 4.9. (B) Number of action potentials increased more steeply with injected current for cells in the Multiple spike category. (C) Peak amplitude declined more gradually with spike number in the Multiple spike category. (D) Both Adapting and Multiple spike cells displayed spike frequency adaptation, but Adapting cells fired at lower frequency (10-15 Hz) on average than Multiple spiking cells (20-25 Hz). (E) Phase plots (dV/dt versus Vm) for 800 msec square pulse stimulation voltage responses of the Single spike, Adapting and Multiple spike cells shown in Figure 4.9. Firing threshold was determined from the phase plot inflection point of the first spike.
staining in control cultures. The poor performance of ChAT antibodies for \textit{in vitro}
labeling of motoneurons has been previously reported (Bohl et al., 2008). In selected
cultures, large cells bodies with prominent neurite branching were observed almost
exclusively. Labeled neurons in control cultures were less uniform and appeared to
mature at a slower rate, possibly due to factors secreted by undesired cell types or
neuron-glia interactions. To our knowledge, this is the first study to demonstrated high
purity mature motoneurons cultures without the need of astrocytes feeder layers.

The functional properties of ES cell-derived motoneurons cultured on mouse
myotubes have been previously investigated (Miles et al., 2004). To determine if
isolated motoneurons maintain similar electrophysiological properties, we performed
whole-cell patch-clamp recordings during maturation of selected motoneurons. High
purity motoneurons displayed transient sodium and potassium currents, as well as
sustained potassium and calcium currents. Both inward sodium and outward transient
potassium currents could by inactivated by pre-pulse steps. Increasing currents were
observed during maturation of motoneurons in response to agonists for ligand-gate ion
channels. Furthermore, selected motoneurons were able to fire multiple action
potentials. These results demonstrate that isolated motoneurons maintain functional
properties. Integration of the Hb9-Puro vector into the mouse genome and subsequent
puromycin selection did not appear to hinder appropriate development of selected
motoneurons.

In this study we demonstrate that Hb9-driven puromycin resistance can be used
as a simple method for purification of ES cell-derived motoneurons. The resulting
population maintains the characteristics of ES cell-derived motoneurons and embryonic
Figure 4.11 Steady-state inactivation of voltage-gated sodium and transient potassium channel currents. (A) Whole-cell currents (control minus TTX) evoked by a step to 0 mV following 60 msec pre-pulses from -100 to +10 mV. (Cs-glucuronate internal solution, d5 after selection). (right) Current-voltage relations for sodium current steady-state inactivation (grey circles) for this cell and for activation (open circles) for the cell in Figure 4.9. Smooth curves are best-fit Boltzmann functions for activation $G = 1 / (1 + \exp(-(V-V_h)/k))$, where $V$ is the step potential in mV, $V_h = -31.7$ mV is the voltage for half-maximal activation and $k = 3.1$ is the slope factor in mV; and, for inactivation $G = 1 / (1 + \exp((V-V_h)/k))$, where $V_h = -52.1$ mV and $k = 7.4$ mV. (B) Whole-cell currents (control minus 4-AP) evoked by a step to 0 mV following 60 msec pre-pulses from -120 to +10 mV (K-glucuronate internal solution, d7 after selection). (right) Current-voltage relations for steady-state inactivation (grey circles) for this cell and for activation of steady-state (open circles) and peak transient (black circles) outward current for the cell in Figure 4.9. Smooth curves are best-fit Boltzmann
spinal motoneurons. This technique may be applied to other systems were yield is low including human ES cell-derived motoneurons, induced pluripotent stem cell-derived motoneurons, and direct conversion of fibroblasts in motoneurons. Further, similar strategies can be applied to other neuronal sub-types with known unique promoters. The resulting near pure population of cells can provide a uniform and consistent platform for drug screening and for the study of motoneuron disease such as amyotrophic lateral sclerosis.
Figure 4.12 Whole-cell currents activated by excitatory and inhibitory agonists. (A) Open boxes indicate periods of exposure to 100 µM kainate (K), NMDA (N, plus 1 µM glycine), glycine (Gly) and GABA (K-glucuronate internal solution, 4d after selection). (B) Current voltage relations for agonist-activated whole-cell currents as membrane potential was ramped from -100 to +100 mV at 1.2 mV/msec. Lines plot the current recorded during agonist exposure minus current in control extracellular solution. Note that currents evoked by inhibitory agonists Gly (teal) and GABA (blue) reverse polarity near -47 mV, consistent with chloride selective channels, whereas the excitatory agonists K (red) and N (gold) reverse near 0mV, as expected for cation selective channels. (Cs-glucuronate internal solution, d4 after selection). (C) Agonist-activated currents increase with time after puromycin selection (n = 7 to 19 cells per time point).
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**Potassium Currents (8)**

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Chapter 5

Summary and Future Direction

5.1 Summary of Findings

The overall goal of this thesis was to develop new tools for stem cell-based therapies, in particular, cell-replacement strategies following spinal cord injury (SCI). Appropriate cell types for replacement must be identified experimentally, requiring large populations of relatively pure cell types. Differentiation of embryonic stem (ES) cells typically results in a heterogeneous population of cells. Included in this mixed population are pluripotent ES cells that can lead to tumor formation following transplantation into the CNS (Erdo et al., 2003). Techniques to purify specific cell-types are needed to realize the full utility of transplant populations. The generation of two high purity cell populations, progenitor motor neurons (pMNs) and post-mitotic motoneurons, was accomplished in this thesis. In addition, the differentiated of high purity progenitor motoneurons was assessed following encapsulation in tissue engineered fibrin scaffolds in vitro and when transplantation into the injured spinal cord.

In Chapter 2, high purity progenitor motor neuron cell populations were generated and characterized using the genetically engineered mouse ES cell line, P-Olig2. Expression of the puromycin resistance enzyme, puromycin N-acetyltransferase (PAC), was accomplished by homologous recombination at the Olig2 locus. PAC expression under the Olig2 gene regulatory elements provided puromycin resistance in pMNs. Puromycin exposure during differentiation of P-Olig2 ES cells killed all cells not
expressing Olig2, resulting in enrichment of Olig2+ pMNs. Due to the rapid
differentiation of pMNs into post-mitotic motoneurons, the latter population was also
enriched. Enriched pMNs differentiated into appropriate cells types including
oligodendrocytes and motoneurons. The results of chapter two demonstrate that
transgenic enrichment provides a powerful tool for generating high-purity neural sub-
type populations.

The ability of high-purity pMNs to survive and differentiate when encapsulated
within tissue engineered fibrin scaffolds in vitro and following transplantation into the
injured spinal cord was assessed in Chapter 3. Delivery of growth factors was
accomplished using a heparin-binding delivery system (HBDS) cross-linked within
fibrin scaffolds. Growth factor presence resulted in small changes in differentiation of
control pMN populations and enriched pMN populations compared to the same cell
population in fibrin scaffolds alone. When the growth factor combination
NT3+GDNF was delivered with the HBDS, significant enhancement of
oligodendrocyte differentiation in enriched pMN populations compared to control
pMN populations was observed. Enriched pMNs survived following transplantation
into the sub-acute SCI. Growth factors did not effect cell survival as previously seen
with heterogeneous ESNPCs. This may be due to removal of undesired pluripotent ES
cells by puromycin selection or differences in the two populations used (pMNs vs.
ESNPCs). Enriched pMNs differentiated into the appropriate cell types in the injured
cord, including cholinergic motoneurons. Transgenic enrichment of pMN transplant
populations appears to be a viable platform for ES cell-based therapies in SCI.
In Chapter 4, the ability to generate high purity neuronal populations, in particular post-mitotic motoneurons, via transgenic expression of PAC was developed and characterized. A new transgenic mouse ES cell line was developed by random incorporation of a vector containing two small enhancer elements of the motoneuron gene Hb9 preceding the minimal β-globin promoter and the PAC gene. Puromycin resistance was specific to post-mitotic motoneurons. High-purity populations of immature post-mitotic motoneurons were obtained. Purity was maintained through maturation and uniform populations of cholinergic motoneurons developed. Mitotic glial populations were not observed at any time point as assessed by proliferation assays and immunofluorescence. High purity motoneurons were electrophysiologically active and expressed appropriate ion channels. This technique provides an inexpensive and more effective method for generating high purity ES-cell derived motoneurons.

Overall the research presented in this thesis demonstrates that antibiotic selection is a reasonable mechanism for generating homogeneous ES cell-derived neural populations. Progenitor and terminally differentiated cell types can be obtained. Following antibiotic selection, isolated cell types behave similar to their *in vivo* counterparts. These cell populations may have therapeutic potential for treating CNS disorders including SCI. Antibiotic selection may advance many studies in developmental biology and provide a uniform platform for toxicology screening.

### 5.2 Recommendations for Future Directions

The work presented in this thesis focused on the development and characterization of high purity neural cell populations. While pMNs and post-mitotic
motoneurons were obtained in these studies, it may be feasible to obtain other cell types and neurons with therapeutic potential including dopaminergic neurons, oligodendrocytes, and spinal interneurons. Antibiotic selection required genomic modification to express the puromycin resistance enzyme. Mouse ES cells were genetically engineered prior to differentiation and needed several weeks to expand in culture. This time scale can be greatly expanded when working with human ES cells. Methods for integration free expression can eliminate the risks associated with transgenic modification and may allow for selection of unmodified cells during differentiation. This technology could facilitate the use of autologous stem cells including iPSCs.

The highly enriched populations of pMNs were evaluated for their ability to survive and differentiation in a short term model of SCI transplantation. To fully determine the tumor forming potential, long term studies are needed. Assessing the long term effect on SCI can elucidate the ability of these cell types to integrate and promote functional recovery and repair. Combining high purity pMNs with other strategies for SCI repair could produce synergistic improvements. Removing undesired cell types can eliminate common complications associated with ES cell-based therapies. Increasing safety can also promote translation into other CNS disorders including amyotrophic lateral sclerosis, Parkinson’s disease, and stroke.

5.2.1 Long-term SCI Studies

We have demonstrated short term survival, differentiation and migration of transplanted high purity pMNs within a sub-acute rodent SCI model. The lack of over-
proliferation by transplanted cells in the presence of growth factors suggests that tumor forming cells have been eliminated by antibiotic selection. Indeed pluripotent ES cells are removed during selection of pMNs with puromycin. However, to fully demonstrate the safety of enriched pMN cell populations, a long term transplantation study is needed. Control unselected cultures should be transplanted to demonstrate that the tumor forming potential previously seen with heterogeneous ESNPC transplant populations is also present in heterogeneous pMN cultures. In addition to tumorigenicity, the ability for high purity ES cell-derived pMNs to integrate into the injured spinal cords can also be assessed at later time points. Behavioral benefit can be demonstrated by the BBB locomotor test and complemented with the grid walk step test. Von Frey hair test should be combined with functional motor tests to determine if pMN transplantation leads to allodynia as commonly observed with NSCs (Hofstetter et al., 2005, Macias et al., 2006).

5.2.2 Other Potential Cell Types

ES cells have been used to generate many different cell types from all three germ layers (Williams et al., 2012). Apoptotic death of oligodendrocytes has been shown following SCI leading to demyelination of spared axonal pathways (Crowe et al., 1997). Many protocols have been developed to enrich ES cell-derived populations for oligodendrocytes (Liu et al., 2000, Sharp et al., 2011). High purity differentiation of mouse and human ES cells has been demonstrated, however, this may depend on the cell line used and cannot guarantee removal of pluripotent embryonic stem cells that fail to differentiate. Development of oligodendrocytes and motoneurons depends on early
expression of Olig2 as both cell types arise from Olig2-expressiong pMNs (Dessaud et al., 2008). However, Olig2 expression is maintained in oligodendrocytes and peaks in a late phase of oligodendrocyte development. Puromycin selection at this later stage may lead to high purity oligodendrocyte populations with known therapeutic potential in both SCI and multiple sclerosis repair.

Parkinson’s disease (PD) is a neurodegenerative disorder marked by loss of dopaminergic neurons in the substantia nigra (Bjorklund et al., 2002). Symptoms of PD include tremors and motor rigidity. ES cell-derived dopaminergic neurons have been shown to alleviate symptoms of PD in animal models (Roy et al., 2006, Yang et al., 2008). Transplantation of ES cells into the human brain carries significant safety risks and represents a significant hurdle in clinical translation. Tyrosine hydroxylase has been used to drive GFP expression in dopaminergic neurons (Donaldson et al., 2005). Tyrosine hydroxylase may be useful in driving puromycin resistance with subsequent purification. This technique may provide high purity dopaminergic neurons with great ease and enhance the potential of PD cell replacement therapies.

The role of spinal interneurons in relaying descending motor signals around the injury site has been demonstrated in multiple studies (Murray and Goldberger, 1974, Bareyre et al., 2004, Rosenzweig et al., 2010). Differentiation of ventral spinal interneurons follows a similar pattern to motoneurons. Small populations of ventral interneurons have been demonstrated during differentiation of mouse ES cells into motoneurons (Wichterle et al., 2002, Harper et al., 2004). However, the low rate of occurrence of these cell types limits their use in therapeutic applications. Isolating spinal interneurons by antibiotic selection may prove as a critical step in the
development of studies involving spinal interneurons including cell replacement therapies and developmental studies.

5.2.3 Safe Integration or Integration Free

Random incorporation of the PAC vector can lead to gene disruption with catastrophic consequences. Placing these vectors in safe harbor sites for integration, such as the Rosa26 locus in mice or the AAV locus in humans can prevent these complications (Perez-Pinera et al., 2012). Recent advances in zing finger integration technology has allowed for genomic insertion at these sites in an efficient manner without the need for homologous recombination. Fully utilization of antibiotic selection may rely on proper integration or methods that do not require genomic modification.

Recent technological developments for deriving iPSCs from somatic cell types have led to integration free gene expression (Bayart and Cohen-Haguenauer, 2013). Avoiding integration can eliminate the need to clone and expand cells with identified integration events. Potential disruption of important gene expression can also be eliminated. Adding vectors that allow for expression of PAC under control of the Hb9 transcription factor can promote puromycin resistance in developing motoneurons during the directed differentiation of ES cells. With this technology, puromycin resistance can be driven in motoneurons differentiated from any stem cell line allowing for quick and effective purification of motoneurons.
5.2.4 Combination Therapies with High Purity pMNs

While the high purity pMN transplants encapsulated in tissue engineered fibrin scaffolds represent a combination therapy in itself, additional factors may improve the benefit of the total therapy. In particular, methods to overcome CSPG inhibition and myelin-associated inhibition could drastically improve the benefit of pMN transplants. CSPGs can be degraded by inject or sustained delivery of chondroitinase ABC (ChABC), a bacterial enzyme that degrades CSPG glycosaminoglycan inhibitory side chains (Yamagata et al., 1968, Bradbury et al., 2002). ChABC administration has also been shown to improve cell migration from transplanted cellular grafts (Hwang et al., 2011). Degrading CSPGs may allow better integration into host tissue by reducing inhibition at the transplant-host interface and promoting axon extension from transplanted pMNs differentiating into neurons. Myelin-associated inhibition can be reduced by the peptide competitive agonist NEP1-40 (GrandPre et al., 2000). Blocking axon inhibition by myelin debris and myelin present in the white matter may improve axon extension from transplanted cells while improving migration into host tissue. Combining ChABC and NEP1-40 together could maximize the potential therapeutic benefit from pMN transplantation.

5.2.5 High Purity Motoneurons in Amyotrophic Lateral Sclerosis

In CNS disorders where extensive tissue damage is not observed, such as amyotrophic lateral sclerosis (ALS), transplantation volumes must be minimalized to prevent transplant associated damage. In spinal cord this is particularly important, as even small injuries can have catastrophic implications. Unlike SCI, only motoneurons
undergo cell death, suggesting that high purity motoneuron transplants would be most effective in restoring function. Transplantation of heterogeneous ES cell-derived motoneuron cultures have been shown to extend out into peripheral nerves in rodent models of spinal motoneuron disease (Deshpande et al., 2006). Survival of transplanted cells into the uninjured spinal cord is significantly greater compared to the injured spinal cord. Transplantation in amyotrophic lateral sclerosis therefore, may be easier to accomplish. However, if survival of high purity transplanted MNs is poor in rodent models of spinal motoneuron loss, the addition of growth factors or a non-hydrolizable form cyclic adenosine monophosphate (cAMP) may enhance survival. High purity pMN and motoneuron ES cell-derived populations represent a promising platform for cell replacement therapies in CNS disease and disorders.
Appendix A

Matlab Code for Immunohistochemical Analysis of Injured Spinal Cords in Chapter 3

SCIanalyser*.m – Quantative Analysis of green fluorescent protein (GFP), DAPI, and differentiation markers. * = differentiation marker

clc
clear
files = dir("*.tif");
dirSize = numel(files);
fileMatrix = {files.name};
gfpSearch = regexp(fileMatrix,'GFP');
stainSearch = regexp(fileMatrix, '*'); % * = differentiation marker (Btub, ChAT, NeuN, O4, GFAP, nestin)
dapiSearch = regexp(fileMatrix, 'Hoe');
gfpSearch = ~cellfun('isempty', gfpSearch);
stainSearch = ~cellfun('isempty', stainSearch);
dapiSearch = ~cellfun('isempty', dapiSearch);

l=1;
for k = 1:dirSize
    if gfpSearch(k) == 1
        gfpMatrix(:,:,l)= imread(files(k).name);
        l=l+1;
    end
end

l=1;
for k = 1:dirSize
    if stainSearch(k) == 1
        stainMatrix(:,:,l)= imread(files(k).name);
        l=l+1;
    end
end

l=1;
for k = 1:dirSize
    if dapiSearch(k) == 1
        dapiMatrix(:,:,l)= imread(files(k).name);
        l=l+1;
    end
end
dirSplit = dirSize/3;

for p = 1:dirSplit
    gfpFile = gfpMatrix(:,:,p);
    stainFile = stainMatrix(:,:,p);
    dapiFile = dapiMatrix(:,:,p);

    threshedGfp = 0.15;
    bwGfp = im2bw(gfpFile, threshedGfp);
    threshedStain = 0.14;
    bwStain = im2bw(stainFile, threshedStain);
    threshedDapi = 0.135;
    bwDapi = im2bw(dapiFile, threshedDapi);

    clusterSize = 250;
    cellArea = cellSize(bwDapi, clusterSize);

    for j = 1:length(bwGfp)
        for i = 1:(numel(bwGfp)/length(bwGfp))
            if bwGfp(i,j)> 0
                bwStain(i,j)= bwStain(i,j);
                bwDapi(i,j)= bwDapi(i,j);
            else
                bwStain(i,j)= 0;
                bwDapi(i,j)= 0;
            end
        end
    end
    dapiCount = cellCount(bwDapi, clusterSize, cellArea);
    gfpPix=find(bwGfp);
    gfpCount=size(gfpPix);
    gfpCount=gfpCount(1);
    stainPix=find(bwStain);
    stainCount=size(stainPix);
    stainCount=stainCount(1);
    DataMatrix(:,p) = {cellArea gfpCount stainCount dapiCount};
end

cellSize.m—Function to determine average nuclei size for nuclei counting

function meanCellArea = cellSize(fileName, clusterSize)
[dapiFile, dapiNum]= bwlabel(fileName, 4);
dapiStats = regionprops(dapiFile, 'Area');
dapiArea = [dapiStats.Area];

idx = find (dapiArea < clusterSize & dapiArea > 25);
no_outliers = ismember (dapiFile, idx);

[smallCells, numSmallCells] = bwlabel(no_outliers, 4);
dapiStatsSmall = regionprops(smallCells, 'Area');
dapiAreaSmall = [dapiStatsSmall.Area];

meanCellArea = mean (dapiAreaSmall);

**cellCount.m – Function to count number of cells in an image**

function [DapiCount] = cellCount(fileName, clusterSize, cellArea)

[dapiFile, dapiNum] = bwlabel(fileName, 4);

dapiStats = regionprops(dapiFile, 'Area');
dapiArea = [dapiStats.Area];

idx = find (dapiArea < clusterSize & dapiArea > 25);
no_outliers = ismember (dapiFile, idx);

[smallCells, numSmallCells] = bwlabel(no_outliers, 4);

idx2 = find (dapiArea > clusterSize);
outliers = ismember (dapiFile, idx2);
[bigCells, numBigCells] = bwlabel(outliers);
dapiStatsBig = regionprops(bigCells, 'Area');
dapiAreaBig = [dapiStatsBig.Area];

numOutlyingCells = round (dapiAreaBig / cellArea);

DapiCount = sum([numSmallCells numOutlyingCells]);
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2008  University Honors Degree
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2010-2012  Volunteer, Writing Workshop for NSF Research Proposal
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Anatomy and physiology science lessons for middle and high school students

2011  Group Mentor, Senior Design, Biomedical Engineering Undergraduate Class
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2009  Teaching Assistant, Discussion Section for Molecular Cell Biology for Engineers
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2007-2012  Tau Beta Pi Engineering Honor Society
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PATENTS


CONFERENCE PRESENTATIONS


