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Sustained Dual Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury Treatment

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Sustained Dual Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury Treatment

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To my family, for without you I would have never had the strength to finish.

ABSTRACT OF THE DISSERTATION

Sustained Dual Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury Treatment

By Thomas Stephen Wilems

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Professor Shelly Sakiyama-Elbert, Chair

Regeneration of lost synaptic connections following spinal cord injury (SCI) is limited due to local ischemia, cell death, and an excitotoxic environment, which leads to the development of an inhibitory glial scar surrounding a cystic cavity. Myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) are major inhibitors to axon growth inhibition found within the glial scar and limit functional recovery. The NEP1-40 peptide competitively binds the Nogo receptor and partially blocks inhibition from MAIs, while chondroitinase ABC (ChABC) enzymatically digests CSPGs, which are upregulated at the site of injury. The first part of this work develops drug delivery systems which provide sustained delivery of both NEP1-40 and ChABC. In vitro studies showed that the combination of ChABC and NEP1-40 increased neurite extension compared to either treatment alone when dissociated embryonic dorsal root ganglia were seeded onto inhibitory substrates containing both MAIs and CSPGs. Furthermore, the ability to provide sustained delivery of biologically active ChABC and NEP1-40 from biomaterial scaffolds was achieved by loading ChABC into lipid microtubes and NEP1-40 into poly (lactic-co-glycolic acid) (PLGA) microspheres, obviating the need for invasive intrathecal pumps or catheters. Fibrin scaffolds embedded with the drug delivery systems (PLGA microspheres and lipid microtubes) were capable of releasing active ChABC for up to one week and active NEP1-40 for over two weeks in vitro. In addition, the loaded drug delivery systems in fibrin scaffolds decreased CSPG deposition and development of a glial scar,

while also increasing axon growth after spinal cord injury in vivo. Therefore, the sustained, local delivery of ChABC and NEP1-40 within the injured spinal cord may block both myelin and CSPG-associated inhibition and allow for improved axon growth.

The second part of this work looked to improve upon previously established therapies using a combination strategy. A variety of single therapy interventions provide small improvements in functional recovery after SCI but are limited due to the multitude of obstacles limiting recovery. Therefore, a multifactorial therapeutic option that combines several single therapies may provide a better chance of improving recovery. To this end, fibrin scaffolds were modified to provide sustained delivery of neurotrophic factors, the sustained delivery of anti-inhibitory molecules, and encapsulation of embryonic stem cell-derived progenitor motor neurons (pMNs). The efficacy of the scaffolds, prior to transplantation, was established by validating pMN viability, migration, and extension of processes was unaffected by culture within scaffolds with sustained delivery of anti-inhibitory molecules. The combination scaffolds were then transplanted into a rat sub-acute SCI model. The anti-inhibitory molecules were capable of removing proteoglycans within the glial scar when embedded in fibrin scaffolds without pMNs included. While pMNs incorporated into fibrin scaffolds without anti-inhibitory molecules showed significant cell survival, differentiation into neuronal cell types, axonal extension in the transplant area, and the ability to integrate into the host tissue, the combination of pMNs with anti-inhibitory molecules led to decreased cell survival and increased inflammation in the lesion site. Thus combination therapies maintain therapeutic potential for treatment of SCI but further work is needed to improve cell survival and limit inflammation.

Chapter 1

Introduction

1.1 Overview

This work aims to create novel treatment options for patients suffering from spinal cord injury (SCI) using combination therapies that enable cell transplantation and the sustained delivery of growth factors and anti-inhibitory molecules. Specifically, this work develops anti-inhibitory drug delivery systems that promote sustained release of bioactive molecules capable of incorporation into previously established tissue engineered scaffolds for SCI. Following the initial spinal cord trauma, a secondary injury consisting of reactive gliosis results in the formation of a glial scar that presents a physical and chemical barrier, which prevents axonal extension into and across the injury site. Key chemical inhibitors to axonal extension are chondroitin sulfate proteoglycans (CSPGs) and myelin-associated inhibitors (MAIs). CSPGs and MAIs inhibit axonal extension by binding to their target receptors on neuronal membranes which activates the Rho/ROCK cascade leading to growth cone destabilization. Both CSPGs and MAIs have been extensively studied in the context of SCI, and potential therapies have been developed to limit their inhibition. Chondroitinase ABC, a bacterially-expressed enzyme, cleaves the inhibitory sugar chains from the core CSPG protein. The inhibitory effects of MAIs can be blocked by treatment with NEP1-40, a small competitive antagonist peptide, which limits the interaction of MAIs with their neuronal cell membrane receptors. ChABC, NEP1-40, and other anti-inhibitory drugs are typically delivered to the injured spinal cord using invasive intrathecal pumps, catheters, or single dose microinjections, which can exacerbate the injury and scarring at the injury site. Therefore, it may be beneficial to develop drug delivery systems that circumvent the need for invasive drug delivery methods.

The first aim of this work focused on determining whether ChABC and NEP1-40 could work effectively together to overcome multiple forms of inhibition and then developing microparticle drug delivery systems capable of sustained release of the biomolecules. To this end, primary embryonic chick and adult rat dorsal root ganglia cells were harvested, dissociated, and cultured on top of CSPGs and MAIs. In order to assess how the combination treatment of ChABC and NEP1-40 affects the growth potential of neurons on inhibitory substrates, varying amounts of ChABC and NEP1-40 were added to the cell media. Neurite extension on inhibitory spots was measured to establish how the treatments influenced the growth potential of neurons on inhibitory substrates present after SCI. Sustained drug delivery vehicles were developed by incorporating ChABC into hollow cylindrical lipid microtubes and NEP1-40 into spherical polymer-based microspheres. Fluorescent labelling of the molecules prior to loading into their respective delivery systems allowed for observation of drug released overtime. To ensure that the delivery vehicle synthesis did not interfere with ChABC or NEP1-40 function, the bioactivity of drug released from the delivery systems was also measured.

The second part of this work focused on translating the developed drug delivery systems into an adult rat thoracic dorsal hemisection model of SCI. A short term animal study was initially performed to evaluate the efficacy of embedding the drug delivery systems into biomaterial scaffolds and transplanting the scaffolds into an acute SCI model. The acute study measured the drug delivery systems' ability to mitigate the development of the glial scar and improve axonal extension two weeks after transplantation. A longer, sub-acute injury model that treated SCI two weeks after the initial injury was also used to allow for stabilization of the glial scar prior to transplantation of the biomaterial scaffold. Using the sub-acute injury model, the drug delivery systems were incorporated into modified fibrin scaffolds containing growth factors and embryonic stem cell-derived progenitor motor neurons (pMNs). The multifactorial scaffolds aimed to repopulate the cells lost following

injury, increase host and transplant cell survival, and limit the inhibitory local environment. In conjunction with the *in vivo* sub-acute injury model, *in vitro* studies were performed to confirm the efficacy of multifactorial scaffolds for cell transplantation. pMNs were grown within biomaterial scaffolds containing the drug delivery systems for two weeks to measure cell survival.

The following introduction will review key points on the development of the glial scar following SCI, the role of CSPGs and MAIs in inhibiting recovery, and current research that focuses on combination therapies to synergistically aid in functional recovery.

1.2 Spinal cord injury

Spinal cord injury (SCI) is a major medical problem affecting 230,000-320,000 Americans, with 12,000 new cases occurring annually (NSCIS, 2012). SCI typically results in partial or complete loss of function below the initial site of injury; leaving thousands of people without the ability to perform basic daily activities. The average age at injury from 2005-2012 was 41.0 years with 80.6% of the injuries occurring among males. The average life expectancy for a 40 year old patient with motor function loss at any spinal cord level is 73.8 years, which drops to 59.7 years for a high cervical injury (quadraplegia) (NSCIS, 2012). Thus, persons with SCI live decades after injury with limited recovery potential.

The estimated lifetime cost of incomplete motor function at any level if the patient was 25 years old at time of injury is over 1.5 million US dollars. Over half of persons with SCI report being employed at the time of injury while only 11.7% report employment one year post injury (NSCIS, 2012). Providing some functional recovery will limit total lifetime costs, allow for increased employment, and most importantly enrich the overall quality of life. Among quadriplegics the most desired functional recovery is arm/hand function followed by sexual function and trunk stability.

Paraplegics mostly responded with sexual function then bowel and bladder control as the highest priority. Therefore, even small degrees of functional recovery can result in large increases in quality of life (NSCIS, 2012).

Spinal cord injuries were long thought to be untreatable and the neurons themselves incapable of regeneration. In 1928, Ramon y Cajal, Nobel Laureate and famous neuroscientist wrote:

> It is for this reason that, once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated (Ramon y Cajal, 1928).

This belief in neuronal rigidity and an intrinsic inability to reform complex neuronal networks in the CNS persisted until the early 1980s when Dr. Albert Aguayo transplanted peripheral nerve grafts into the injured thoracic spinal cord of adult rats (Richardson et al., 1980). Aguayo showed that neurons originating in the CNS entered and extended through the grafts, but the regenerating fibers abruptly stopped upon reentering the CNS and seemed to lose their potential for growth. It was then postulated that either the growth substrate and neurotrophic factors found within the peripheral nerve grafts promote growth or that the growth substrate and chemical factors found within the CNS inhibit growth. In the following decades, research focused on these two original hypotheses, which led to a rise in many exciting research fields and specialties that all aim to understand the factors impeding spinal cord regeneration and to develop methods to mitigate these factors (Schwab and Bartholdi, 1996).

Understanding the pathological mechanisms following SCI and how they affect the acute, sub-acute, and long-term local cellular environment is necessary before useful therapeutic treatments can be developed. To this end, research has shown the initial mechanical trauma can sever axons, damage vasculature, and promote a secondary injury that propagates cell death and leads to a cystic

cavity or cellular void several times larger than the initial trauma. Formation of the cystic cavity results from a spectrum of responses from local cells and infiltrating cells, which triggers the formation of an inhibitory scar. The fibrotic scar, termed the glial scar, forms a strong chemical and physical barrier between the cystic cavity and surrounding tissue. The glial scar severely limits the natural regeneration potential within the spinal cord, prompting many therapeutic strategies that focus on overcoming inhibitory aspects of the glial scar. The events leading to the development of the glial scar surrounding the injury site following SCI are reviewed below.

1.2.1 Secondary Injury Following SCI

All human spinal cord injuries are unique and typically occur alongside multiple other traumatic injuries making them difficult to treat. The diversity of injuries that lead to SCI clinically also means that a variety of animal models are necessary for determining the underlying mechanisms that are common between injury models. Typically injuries are classified according to the segmental level along the spinal cord, the type of injury (i.e. weight drop, balloon compression, or axotomy), and the completeness of the injury. This categorization allows for comparisons between the varying injury models and enables researchers to determine which mechanisms play the most significant roles. In many animal models the initial injury leads to both local and systemic changes including ischemia, edema, release of toxic molecules, peripheral cell infiltration, and activation of microglia. These events are interconnected and complicate the investigation of their roles following SCI. The main secondary mechanisms following the initial trauma will be reviewed below; it is important to note that each mechanism discussed is part of a larger more dynamic picture that results in the formation of the glial scar.

Vascular Mechanisms

Immediately following injury, hypoperfusion of the central gray matter is consistently seen while white matter is believed to be more resilient to vasculature changes (Kobrine et al., 1975, Ducker et al., 1978a, Ducker et al., 1978b, Rivlin and Tator, 1978, Holtz et al., 1989). The immediate reduction in blood flow results in a substantial loss of oxygen delivery to the injured tissue, as well as an inability to remove toxic cues released into the injury site (Ducker et al., 1978b, Hayashi et al., 1980, Stokes et al., 1981). Compounding the decrease in blood flow, the instantaneous release of blood into the spinal cord results in edema and hemorrhages in the gray matter that can spread to the white matter from 1 hour to 1 week following injury (Green and Wagner, 1973, Noble and Wrathall, 1989, Lemke and Faden, 1990, Tator and Koyanagi, 1997).

Within a few hours post-injury, swelling and vacuolation of vascular endothelial cells in both white and grey matter are observed, indicating ischemic conditions in the injured cord. Edema or swelling may cause additional injury by increasing compression on the surviving tissue and by contributing to an unfavorable electrolyte balance within the extracellular environment, which is normally highly regulated to support neuronal function (Casella et al., 2002). Strategies to overcome changes in vascular architecture following SCI have shown the important role the microvasculature play in the secondary injury (O'Carroll et al., 2008, Saadoun et al., 2008, Fassbender et al., 2011).

Ionic and Excitotoxic Mechanisms

Ionic imbalances from hemorrhages, ischemia, and edema contribute to loss of function and necrosis. Relatively minute changes in the ionic balance can lead to a loss of impulse conduction through intact nerve fibers, while the burst release of excitatory molecules may lead to further cell death. Loss of impulse conduction may be a potential reason for spinal shock, a condition in which a state of paralysis is seen in the muscles below the injury level. Spinal shock is not fully understood,

but the duration of the symptoms has been correlated to the severity of the injury and has been seen in both animal and human injuries (Bach-y-Rita and Illis, 1993).

The initial trauma and cell death within the spinal cord releases the excitotoxic chemicals glutamate and aspartate, which have been shown *in vitro* to cause accumulation of intracellular Na^+ and Ca^{2+} ions (Panter et al., 1990, Liu et al., 1991, Choi, 1992). Ionic imbalances in Na^+, K^+, and Ca^{2+} concentrations lead to cell swelling and death, which releases more excitotoxic chemicals into the surrounding area causing a swath of death that expands outward from the injury epicenter (Lewin et al., 1974, Anderson et al., 1976). Increased intracellular Ca^{2+} concentration also affects many cellular processes not directly related to cell signaling, such as breakdown of the cellular membrane and the production of free radicals that exacerbate tissue injury, by triggering a more pronounced inflammatory response (Rasmussen, 1986, Demediuk and Faden, 1988, Woodroofe et al., 1991). NMDA receptors have been implicated as mechanisms for increased intracellular calcium accumulation due to the voltage-gated channels being persistently active. NMDA receptor blockers have been used to reduce intracellular calcium accumulation and limit cell death following injury (Faden et al., 1988, Gomezpinilla et al., 1989, Sun and Faden, 1994).

Inflammatory Response

SCI triggers a robust inflammatory response that begins hours after injury, peaks during the following week, and plays a role in long term recovery after injury. After injury, astrocytes and microglia enter a reactive state, indicated by alterations in their morphology and gene expression profiles; in this state they recruit inflammatory cells through expression of cytokines, such as interlekin-1beta and tumor necrosis factor-alpha, which peaks within hours after injury (Pineau and Lacroix, 2007). The first immune cells to enter the CNS following injury are neutrophils, whose main function is to destroy foreign bacteria that may have been introduced during injury, although in

animal models the typical SCI occurs under sterile conditions (Means and Anderson, 1983, Travlos et al., 1994, Carlson et al., 1998, Fleming et al., 2006, Donnelly and Popovich, 2008). The next cell recruitment period occurs 3 days post-injury when macrophages infiltrate the injured cord (Stirling and Yong, 2008). Macrophages are not native to the CNS and enter the spinal cord through the leaky vasculature, but once in the CNS the macrophages are difficult to distinguish from reactive microglia (Nathan et al., 1980, Celada and Nathan, 1994). Both neutrophils and macrophages generate oxygen free radical species and lysosomal enzymes that cause non-specific degradation of the surrounding cells and tissue (Cassatella, 1995, Taoka and Okajima, 2000).

Limiting the initial immune reaction using antibodies against integrins specific to pro-inflammatory cytokines, led to decreased tissue damage at early time points post-injury (Gris et al., 2004, Bao et al., 2005). It is important to note that the inflammatory response may be a double-edged sword that relies on both the phagocytic and growth promoting properties of macrophages. Macrophages are one of the few cell types capable of phagocytosis of myelin-debris, which has been implicated as a major barrier to axonal growth following SCI. Macrophages also secrete a diverse set of molecules that can elicit many biological responses and promote a return to homeostasis (Fahey et al., 1990, Clark, 1993). Following the initial trauma and secretion of pro-inflammatory cytokines, the resident and recruited macrophages activate differentiation expression patterns that direct them into inflammatory M1 or anti-inflammatory and neuroprotective M2 subtypes (Auffray et al., 2009, Shechter et al., 2009, London et al., 2011).

The complex mechanisms following SCI have all been shown to affect tissue degradation and mitigation of the vascular, ionic, or inflammatory damage results in improved recovery by limiting the loss of functional synaptic connections. Weeks to months after the primary injury, the secondary injury is stabilized and results in the formation of a glial scar that separates surviving tissue from the cystic cavity.

1.2.2 Glial Scar Formation and Inhibitory Cues

The secondary events following the initial trauma are complex, interconnected and result in an area of injury several times larger than the initial injury site. The rapid necrosis seen following SCI stabilizes over several weeks and results in the formation of a glial scar surrounding a cystic cavity. The main contributors to the glial scar are astrocytes but microglia, macrophages, neutrophils, fibroblasts, and other infiltrating peripheral cells have also been seen (Wakefield and Eidelberg, 1975, Means and Anderson, 1983, Blight, 1985, Giulian et al., 1989, Maxwell et al., 1990, Xu et al., 1990, Abnet et al., 1991, Dusart and Schwab, 1994). Astrocytes at the site of injury have altered genetic profiles and undergo morphological changes resulting in the formation of reactive astrocytes (Gearhart et al., 1979, Kruger et al., 1986, Maxwell et al., 1990, Bunge et al., 1994, Dusart and Schwab, 1994). Cytokines and soluble factors are implicated as a driving force for conversion into the reactive cell state (Panter et al., 1990, Woodroofe et al., 1991, Lindholm et al., 1992, Logan et al., 1992, Taupin et al., 1993, Tchelingerian et al., 1993). Injection of interleukin-1 into the uninjured spinal cord was able to trigger reactive gliosis (Giulian et al., 1988). Reactive astrocytes demonstrate significantly upregulated expression of glial fibrillary acidic protein (GFAP), which is critical for the formation of intermediate filaments. A hallmark of the glial scar is a reactive astrocyte "net" that fully surrounds the cystic cavity via the extension of their end feet to form a multilayer interface separating healthy and necrotic tissue.

The glial scar not only serves as a physical barrier to neurite growth but astrocytes also express a multitude of ECM proteins that provide inhibitory chemical cues to extending axons. The ECM within the glial scar mainly contains fibronectin, laminin, N-CAM, thrombospondin, heparin sulfate proteoglycans, chondroitin sulfate proteoglycans (CSPGs), and tenascin (Grierson et al., 1990). Although some components of the ECM have neurite growth promoting properties, significant upregulation and deposition of CSPGs has shown to be a potent inhibitor to neurite

growth. CSPGs (aggrecan, neurocan, brevican, and phosphocan) consist of several highly sulfated glycosaminoglycan (GAG) side chains connected to a core protein. CSPGs are thought to provide important axon guidance cues during development and play a key role in stabilizing synaptic connections through perineuronal nets (Knudson and Knudson, 2001, Pizzorusso et al., 2002, Hwang et al., 2003, Laabs et al., 2005, Sirko et al., 2007). GAG side chains vary in length and contain unbranched polysaccharides with repeating D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) disaccharide units that can be sulfated at the C2 position of GlcA and at the C4 or C6 position of GalNAc. The sulfation patterns of the GAG side chains lead to significant changes in axon guidance cues produced by the CSPGs (Wang et al., 2008).

CSPGs play a multifaceted role during development and in the mature adult, so it is not surprising that CSPGs affect a multitude of receptors found on neurons. For instance, GAG side chains can bind to PTPσ/LAR type receptors and NgR1/NgR3 receptors found on neuronal cell membranes and trigger the Rho/ROCK cascade leading to microtubule destabilization and growth cone collapse (Shen et al., 2009, Fisher et al., 2011, Dickendesher et al., 2012). CSPGs can also interact with the transmembrane protein semaphoring 5A to convert them from attractive to repulsive guidance cues (Kantor et al., 2004). Furthermore, CSPGs limit the interaction of integrins with growth promoting ECM proteins such as laminin. Increasing the amount of available integrins on neuronal cell membranes can overcome CSPG inhibition by increasing the probability that the integrins bind to their target ECM proteins (Condic et al., 1999, Afshari et al., 2010, Tan et al., 2011). The degradation of the GAG side chains from the core protein using enzymatic degradation with chondroitinase ABC (ChABC) leads to significant increases in neurite and axon extension *in vitro* and *in vivo*, while also improving functional recovery (Bradbury et al., 2002b). Therefore, limiting or degrading the ability of CSPGs deposited by reactive astrocytes to bind target receptors can be a potent therapeutic strategy for SCI.

It is important to note that astrocytes have a dual nature and provide necessary functions for recovery through secretion of soluble factors and deposition of growth promoting substrates. Neurites are consistently seen growing over astrocyte bridges into and around the injury site (Taylor et al., 2006b, Zukor et al., 2013, Goldshmit et al., 2014). *In vitro* studies have shown astrocyte cultures to be highly growth promoting substrates for neurite extension (Noble et al., 1984). One key difference between the growth promoting and growth inhibiting substrates is the presence of CSPGs, where growth promoting substrates lack CSPGs. Interestingly, the immature CNS has a higher potential for recovery after SCI and typically has less pronounced reactive gliosis (Smith et al., 1986, Rudge and Silver, 1990). This suggests that maturation of astrocytes plays an important role in deciding if astrocytes become reactive after injury. It is possible that mature astrocytes respond differently to released cytokines or other soluble factors compared to immature astrocytes. Therefore it may be possible to drive astrocytes into a more growth permissive state instead of the predominant inhibitory reactive state. This has been studied with some success; the delivery of neural growth factor (NGF) into adult rat CNS led to an increase in cholinergic fibers penetrating into grafts by growing along astrocyte formed ECM (Kawaja and Gage, 1991). The enhanced fiber growth may partially be attributed to effect of NGF on astrocyte deposition of growth promoting substrates.

The glial scar and CSPGs account for a significant proportion of inhibition following SCI but several other factors also influence neurite extension. One powerful growth inhibitor is myelin debris that remains within the injury site and non-phagocytosed. In the CNS, myelin formation begins at the end of development when oligodendrocytes differentiate into mature states. Upon injury, myelin debris remains in and around the injury site due to neuronal and glial cell death, as well as neuronal die back (Savio and Schwab, 1989, Bandtlow et al., 1990, Akbik et al., 2012). The myelin debris and its MAIs can be phagocytosed by microglia, macrophages, and infiltrating

Schwann cells, but MAIs persist in high concentrations around the injury site. White matter, which contains increased amounts of myelin, is significantly inhibitory to neurite extension compared to grey matter, and isolated CNS myelin substrates provide a highly non-permissive substrate *in vitro* (Savio and Schwab, 1989).

The myelin-associated membrane proteins, such as Nogo-66, MAG, and OMgp bind the Nogo receptor on neurons, which triggers the Rho/ROCK cascade through the transmembrane proteins Lingo-1 and p75 (Domeniconi et al., 2002b, Liu et al., 2002, Wang et al., 2002a, McGee and Strittmatter, 2003). Blocking the upstream activation of the ROCK signaling pathway can be achieved using Nogo receptor fragments, targeted Nogo receptor antibodies, or inactivating downstream effectors of the pathway (Liebscher et al., 2005c, Forgione and Fehlings, 2014). Similarly, limiting the activation of RhoA, RhoB, and RhoC proteins increased functional recovery in animal studies (Dergham et al., 2002, Fournier et al., 2003). Delayed treatment with Rho-kinase inhibitors did not show improved recovery after injury, which suggests a need for acute or semi-acute therapies after SCI (Nishio et al., 2006). Recent research has also implicated myelin associated lipids as inhibitors to neurite extension (Mar et al., 2015). Interestingly, the researchers showed that myelin lipids, cholesterol and sphingomyelin, inhibit axon growth and act through a Rho/ROCK independent pathway.

The timing and duration of treatment options is an important factor that has not yet been addressed in this thesis but may be a major contributor to recovery. For instance, recent research has shown that the duration of ChABC treatment in cat hemisection models led to differences in functional recovery and that longer treatment times increased the number of rubrospinal tract neurons below the lesion site (Mondello et al., 2015). Furthermore, the combination therapy of ChABC and anti-Nogo-A was coupled with treadmill training in a rat dorsal column crush injury with varying results. The researchers measured improved functional recovery when anti-Nogo-A

was infused into the injury site immediately after injury and continually for two weeks, while ChABC treatment began three weeks after the injury and lasted for 10 days. The researchers noted that if the ChABC treatment was overlapped with anti-Nogo-A immediately after injury or the anti-Nogo-A had a delayed start to treatment that no further functional recovery was seen compared to single treatments alone (Zhao et al., 2013). Therefore, the timing and duration of treatments may be an important factor that is not often studied within spinal cord injury. Also, determining how single treatment options interact with each other during combination treatments may provide varying results whether the treatments are sequential or overlapped.

In conclusion, the glial scar arises to limit the expansion of the secondary injury and forms a barrier between healthy and necrotic tissue. After stabilization it is a formidable obstacle to recovery because of its robust inhibitory cues that destabilize the growth potential of extending axons. The following sections will detail the main areas that research has focused on to overcome the multitude of obstacles present after SCI.

1.3 Current treatment options for SCI

Since Aguayo showed that CNS neurons were capable of long distance regeneration through PNS grafts, SCI research has led to an array of treatments that target various obstacles that arise. The main goal of any therapeutic approach is to reform lost synaptic connections, but the therapies vary in how to meet this fundamental goal. Some therapies aim to boost the intrinsic growth potential of surviving neurons while others aim to limit the inhibitory molecules found in the glial scar. Therapies can also aid in recovery through cell transplantation of specific cell types, again with the goal of aiding host cells in reforming synaptic connections. Several treatments have bridged the gap from animal to human studies, but few human trials have been successfully completed due to

poor results or loss of funding. The two main treatment areas are molecular or drug based therapies and cell transplantation therapies.

1.3.1 Molecular Therapeutic Strategies

Various drugs have been tested in animal models for their potential to overcome barriers limiting recovery following SCI. Some therapies deliver molecules that increase the intrinsic growth potential of neurons or increase tissue sparing following SCI. Many of the molecular therapies work by removing, limiting, or masking the inhibitory environment from regenerating neurons, including cues derived from CSPGs and MAIs. These were briefly discussed in section 1.2.2 pertaining to secondary injury and development of the glial scar. Below we will review the main molecular therapies studied for use in SCI treatment in greater detail.

1. Chondroitin Sulfate Proteoglycans

Under normal conditions CSPGs are major components of perineuronal nets (PNNs) and are found within CNS extracellular matrix (Kwok et al., 2011, Kwok et al., 2012). CSPGs are significantly upregulated following SCI with peak deposition between 10 to 14 days post injury and form a dense chemical barrier to axon growth (Jones et al., 2003). Much of the inhibition from CSPGs is due to the presentation of the GAG side chains and their interaction with target receptors on neuronal cell membranes.

Treatment of CSPGs with ChABC degrades the GAG chains and prevents the activation of the receptors on the neuronal cell membrane. ChABC is a bacterial derived enzyme isolated from *Proteus vulgaris* and degrades the GAG chains into soluble disaccharides leaving the core stub protein (Yamagata et al., 1968). Not only is the resulting chondroitin sulfate E-disaccharide growth promoting, but ChABC mediated degradation results in the release of bound growth factors from

CSPGs (Rolls et al., 2004). The efficacy of ChABC to remove inhibition from CSPGs has been shown in several *in vitro* and *in vivo* studies resulting in increased axonal sprouting and regeneration, a decrease the glial scar area, and improved functional recovery in acute and chronic injury models (Zuo et al., 1998, Moon et al., 2001, Bradbury et al., 2002b, Garcia-Alias et al., 2008, Garcia-Alias et al., 2009, Wang et al., 2011).

In most studies, ChABC is bacterially expressed, which can complicate translation into clinical use, due to the risk for enhanced immune response. Recently, an engineered mammalian-compatible ChABC was used in a rat thoracic contusion injury and showed the potential for improved axonal sprouting and functional recovery; the ChABC was delivered using lentiviral vectors for continual expression of ChABC and large-scale digestion of CSPGs (Bartus et al., 2014). The continual degradation of CSPGs within the CNS may be counterproductive on a larger time scale because of the inability to reform perineuronal nets, which are vital for stabilizing synaptic connections between axons and dendrites.

CSPGs target many receptors located on the neuronal cell surface but have shown a high affinity for PTP σ and LAR type receptors (Dyck et al., 2015). Research from Dr. Jerry Silver's lab removed the need for using the highly labile ChABC and invasive catheters by subcutaneous injection of a peptide that blocks binding of GAG chains to the PTP σ receptor. The peptide was shown to localize to the site of a thoracic contusion injury and increased axonal sprouting, regeneration, and functional recovery compared to a non-active control peptide (Lang et al., 2015).

2. Anti Myelin-Associated Inhibitors

MAIs, such as Nogo-A, OMgp, and MAG, are important chemical cues in mature CNS that limit axon growth and plasticity to stabilize functioning neuronal circuits. Unfortunately, following SCI MAIs are not degraded or removed from the injury site causing limited sprouting and

regeneration. The main MAIs interact with neurons through a common receptor, Nogo receptor (NgR), which initiates the Rho/ROCK cascade, resulting in microtubule disassembly and growth cone collapse (Domeniconi et al., 2002a, Liu et al., 2002, McGee and Strittmatter, 2003). Currently three types of Nogo receptors (NgR1, NgR2, and NgR3) have been characterized; Nogo, OMgp, and MAG are ligands for NgR1 and NgR3 but only MAG interacts with NgR2 (Venkatesh et al., 2005). Although MAIs have been shown to bind to other cell membrane receptors, blockage of NgR1 using a competitive antagonist, function-blocking antibody, or soluble NgR1 ectodomain significantly improved neurite outgrowth on myelin substrates and deletion of NgR1, but not NgR2, led to increased regeneration of injured optic nerves in rats (GrandPre et al., 2002, Liebscher et al., 2005b, Maier et al., 2009, Dickendesher et al., 2012). Therefore, targeting of the NgR1 receptor is necessary and sufficient in limiting inhibition from MAIs following injury.

Delivery of Nogo-A function-blocking antibodies via intrathecal pump in a rat thoracic injury model resulted in long distance regeneration of corticospinal tract axons and improved functional recovery (Liebscher et al., 2005b). Interestingly, the combination treatment of ChABC delivered via catheter and anti-Nogo-A delivered via intrathecal pump significantly improved axon sprouting, regeneration, and functional recovery over either treatment alone when coupled with rehabilitative treadmill training (Zhao et al., 2013). However the delivery of the molecular therapies could be improved by removing the need for catheters and intrathecal pumps, which cause increased scarring and injury at the site of injury (Jones and Tuszynski, 2001).

3. Growth factors and therapies that enhance intrinsic growth potential

The cell loss and expansion of the secondary injury following SCI can be reduced by delivering neurotrophic factors and molecules that enhance the intrinsic ability of axons to regenerate. Much of this introduction has discussed the need to overcome the inhibitory local

environment caused by formation of the glial scar, but adult neurons show significantly less growth potential than embryonic or developing neurons and require additional stimuli to prompt regeneration. Thus, research has focused on treatments that increase the intrinsic growth potential of adult neurons in an attempt to revert them into a more proliferative embryonic state. It is likely that therapies that positively affect the intrinsic growth capacity of neurons combined with therapies that limit inhibition from extrinsic sources will provide improved treatment than either option alone.

Neurotrophic factors are normally secreted by local glia and are vital to the maintenance of healthy tissue. Following injury, specific growth promoting factors are upregulated within the spinal cord and help to mitigate cell loss. Exogenous delivery of a variety of neurotrophic factors has also been shown to increase cell survival and limit formation of the glial scar. Brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are neurotrophic factors that have demonstrated promising effects in many studies when delivered into the injured spinal cord (Schnell et al., 1994b, Grill et al., 1997a, Jakeman et al., 1998, Liu et al., 1999). Interestingly, BDNF appears to preferentially promote regeneration of rubrospinal, raphespinal, cerulospinal and reticulospinal tracts, while NT-3 affects regeneration of the corticospinal tract and dorsal sensory axons (Ye and Houle, 1997, Menei et al., 1998, Kerr et al., 1999, Storer et al., 2003, Taylor et al., 2006a). BDNF treatment was shown to increase axon regeneration and synapse formation in motor neurons below the injury site but no improvement or decline in functional outcome was measured (Lu et al., 2005, Lu et al., 2012). Delivery of nerve growth factor (NGF) via transplantation of genetically modified fibroblasts for continual secretion of NGF led to increased axonal growth into the transplant area; however, no axons were seen exiting the transplant. This was likely due to the over-abundance of NGF expressed within the graft, resulting in the "candy store effect" whereby axons grow up to the source of growth factor but become unwilling to exit into the non-permissive glial scar (Grill et al., 1997b).

NGF, BDNF, and NT-3 affect neurons by binding to the p75 neurotrophin receptor and modulating TrkA, TrkB, and TrkC signaling; other growth factors affect glia in the CNS instead of neurons. For example, glial cell-line derived neurotrophic factor (GDNF) is expressed by both astrocytes and oligodendrocytes and activates GFRα receptors located on neurons, oligodendrocytes, astrocytes, and Schwann cells (Jaszai et al., 1998, Hoke et al., 2003, Zhang et al., 2009, Cristante et al., 2012). GDNF delivered into the injured spinal cord consistently resulted in increased myelination of regenerating fibers, enhanced sensory fiber axon growth and motor neuron growth (Blesch and Tuszynski, 2003). However GDNF also promotes growth of pain fibers after injury, which may lead to negative side effects and limit its potential use as an SCI treatment (Tuszynski et al., 1994, Blesch and Tuszynski, 2003, Ramer et al., 2003). Delivery of platelet-derived growth factor (PDGF) can aid stabilization and maturation of blood vessels at the injury site and is an important factor in oligodendrocytes maturation (Raff et al., 1988, Almad et al., 2011). Previous research from the Sakiyama-Elbert lab looked at the combination of promising growth factors to determine which combination led to the greatest increase in neuronal regeneration and decreased glial scar formation (McCreedy et al., 2014). The combination of NT-3 and PDGF growth factors increased the presence of neuronal markers both rostrally and caudaully to the injury site and improved migration of transplanted cells into the host spinal cord. These studies have shown the benefits of using neurotrophic factors to enhance the growth potential of axons and possibly mitigate the inhibitory extrinsic environment to overcome the limitations affecting recovery following SCI.

Enhancing the regenerative potential of adult neurons can also be achieved without the use of neurotrophic factors. Recent research promoting activation of intracellular pathways that lead to growth cone stabilization through microtubule assembly has shown promise (Cai et al., 2001, Liu et al., 2010, Blesch et al., 2012, Walker et al., 2012, Ma et al., 2014). Continuous delivery of cyclic

adenosine monophosphate (cAMP) into the injured spinal cord increases activation of the mTOR pathway and promotes axon extension. cAMP is a secondary messenger that is upregulated during embryonic stages of development but is downregulated in adult populations. Furthermore, the addition of cAMP to primary cells cultured on growth permissive or inhibitory substrates led to increased neurite extension. The regrowth potential of adult corticospinal neurons is believed to decrease after maturation and is accompanied by a downregulation of the mTOR pathway. The deletion of PTEN, a negative regulator of mTOR, showed enhanced sprouting of injured corticospinal neurons in mice and led to successful reformation of lost synaptic connections distal to the injury (Liu et al., 2010).

All three molecular therapy areas have shown significant benefits to recovery after injury, but combination therapies that provide both neuroprotection and affect extrinsic and intrinsic factors will be needed to further enhance functional recovery.

1.3.2 Cell Replacement Therapies

One major research strategy following SCI is cell transplantation to aid in replenishing cells lost. A wide variety of cell types with unique therapeutic strategies have been investigated as treatment options. Some cells, such as Schwann cells, have been shown to express a multitude of factors and remove some extrinsic barriers, while other cell types, such as bone marrow stromal cells and fibroblasts, are autologous populations with high growth rates and are relatively easy to acquire. A large emphasis has also been placed on transplanting cells that are relevant to the CNS, such as astrocytes, olfactory ensheathing glia, or neural progenitor cells. One large benefit of cell transplantation as a therapy is the ability to genetically alter cells to produce specific proteins that can also aid in recovery. A vast amount of research published within the last decade has combined

cell transplantation with the molecular therapeutic strategies discussed in section 1.3.1. The main cell types used in transplantation studies and their combination therapies will be reviewed below.

1. Schwann Cells

Schwann cells are found in the PNS and provide neurotrophic support and myelination to extending axons. Following PNS injury, Schwann cells are capable of dedifferentiating from a more mature state to an immature state that allows phagocytosis of myelin debris, unlike oligodendrocytes in the CNS. In addition to removing inhibitory myelin debris, Schwann cells express a wide range of growth factors including NGF, BDNF, and GDNF that were previously discussed in section 1.3.1. Following SCI, Schwann cells infiltrate the CNS through the ventral roots and can remyelinate axons in spared white matter (Bresnahan et al., 1976, Bunge et al., 1994, Beattie et al., 1997, Bruce et al., 2000).

Transplantation of autologous Schwann cells alone has shown improved regeneration and functional recovery in a few studies (Takami et al., 2002, Barakat et al., 2005, Schaal et al., 2007). One major limitation to the use of autologous Schwann cells is the invasiveness of harvesting the cells from peripheral nerves (Casella et al., 1996, Hilton et al., 2007). However the technique remains at the forefront of research and further improvements have been measured with the transplantation of genetically engineered Schwann cells that express neurotrophic factors and ChABC to improve engraftment of transplanted cells, increase myelination and neuronal extension, decrease glial scar development, and improve functional recovery (Kanno et al., 2014). Significant Schwann cell research has been performed by the Bunge lab at the Miami Project to Cure Paralysis, which is currently enrolling patients for phase 1 clinical trials of autologous Schwann cell grafts for treatment of acute and chronic SCI. Although the hope is that the clinical trials successfully show the efficacy

of Schwann cell for SCI treatment, future trials may require multifactorial therapies that also deliver neurotrophic factors or mechanisms to limit the inhibitory environment found in the glial scar.

2. Olfactory Ensheathing Glia

Olfactory ensheathing cells (OECs) are another peripheral nervous system glial cell that infiltrates the CNS following traumatic injury. OECs aid in regeneration of transected or injured olfactory bulbs by providing a growth permissive substrate for peripheral olfactory axons to migrate into the CNS and reform lost synaptic connections. Similarly, transplantation of OECs into the spinal cord promotes regeneration of transected dorsal root axons, corticospinal tract axons, and serotenergic axons by providing myelination and cellular bridges across the inhibitory lesion, although functional improvements have been limited (Ramoncueto and Nietosampedro, 1994, Li et al., 1997, Ramon-Cueto et al., 2000). Some reports indicate that transplantion of olfactory nasal mucosa cells, a common source of OECs, leads to increased motor recovery following complete transections, but replication studies failed to observe the same results.

As with Schwann cells, the combination of OECs with other treatments further enhances their therapeutic potential. Combining OEC transplantation with rehabilitative treadmill training after a complete thoracic transection showed improved hindlimb functional recovery (Lu et al., 2001, Lu et al., 2002, Steward et al., 2006). More recently OECs garnered national media attention when a group of European scientists transplanted OECs into a 40 year old male with a transected thoracic spinal cord injury resulting from a knife wound 4 years earlier (Tabakow et al., 2014). The patient is reported to have regained functional recovery following transplantation although the treatment has undergone scrutiny for its claims that the OECs are the reason for the functional recovery (Guest and Dietrich, 2015). While OECs do appear to have some therapeutic potential, it is unclear whether that potential outweighs the benefit of using other more easily attainable autologous cell types.

3. Bone Marrow Stromal Cells

Bone marrow stromal cells (BMSCs) are an interesting cell type used for SCI treatment because they are not naturally found in the PNS or CNS following injury, but the transplanted cells are capable of expanding to fill the cystic cavity and secrete growth factors that aid in recovery. The efficacy of BMSCs has been debated; some research has shown functional improvement, but replication studies failed to duplicate the positive results (Chopp et al., 2000, Hofstetter et al., 2002). Despite the concerns over efficacy, BMSC transplants in clinical trials showed a decrease in lesion size and increase in neuroprotection and sprouting, although BMSCs do not differentiate into neurons and therefore any recovery is an indirect effect. BMSCs are derived from the hematopoietic cell fraction of bone marrow and are purified by adhesion to plastic. This results in a heterogeneous mixture of hematopoietic stem cells and mesenchymal stem cells with high variability between laboratories, which may explain the differences between studies. Human BMSCs derived from four different donors had variable results when transplanted into a rat hemisection SCI model, highlighting the importance of better understanding and characterizing the types of cells being transplanted at the injury site (Neuhuber et al., 2005). However most clinical trials involving cell transplantation therapies for SCI have used BMSCs due to them being autologous cells with relatively easy harvesting and implantation but the treatments again were split when showing functional improvements (Sanchez-Ramos et al., 2000, Hofstetter et al., 2002, Krabbe et al., 2005, Chernykh et al., 2007, Phinney and Prockop, 2007, Song et al., 2007).

4. Neural Stem Cells

Neural stem cells (NSCs) are a highly studied cell type for SCI treatment and can be derived from several different autologous and non-autologous sources. While other cell transplantation therapies provide trophic support to surviving local cells or deposit ECM to bridge the cystic cavity,

NSCs are unique because they are precursor cells that can mature to replace lost cell populations including oligodendrocytes, astrocytes, and neurons. Transplanted NSCs from fetal spinal cord tissue have been shown to fill the cystic cavity and form functional connections with the surviving host tissue in several animal models (Houle and Reier, 1989, Reier et al., 1992). However, research using fetal spinal cord tissue has been hindered due to ethical and political reasons, which may limit clinical translation of the treatment. Future NSC treatments may rely on human NSC lines that have been immortalized or derived from autologous sources and induced into a neural fate but autologous induced pluripotent stem cells pose risks of spontaneously reverting back into immature domains (Choi et al., 2014). Protocols were recently established to derive NSCs from differentiated murine fibroblasts; retrovirus vectors were used to generate induced pluripotent stem (iPS) cells, which were then driven into an NSC fate using pro-neural growth factors and media in suspension culture (Moon et al., 2011). The use of iPS cells is promising but limitations arise due to long NSC generation times which are at least 2 months.

Transplantation of NSCs into the spinal cord typically results in the differentiation of glia over neurons. This phenomenon led to the use of more restricted progenitor domains for transplantation termed neuronal restricted precursors (NRPs) and glia restricted precursors (GRPs), which can be purified from each other using immunopanning for specific cell surface markers (Cao et al., 2001, Cao et al., 2002). NRPs are typically not used following SCI due to a very poor survival rate following transplantation (Cao et al., 2002). GRPs preferentially differentiate into astrocytes following transplantation, although increased myelination and oligodendrocyte formation has been observed (Mitsui et al., 2005b, Karimi-Abdolrezaee et al., 2006, Neuhuber et al., 2008, Parr et al., 2008). Research involving transplantation of primary or NSC-derived astrocytes is promising due to the formation of astroglial bridges that provides a substrate for axonal extension and cell migration.

5. Embryonic Stem Cells

Embryonic stem cells (ESCs), such as NSCs, are strong therapeutic candidates that can replace lost cell populations with phenotypically similar cells. ESCs can be continually expanded and can differentiate into cells from all three germ layers. ESCs induced into a neural progenitor fate (ES-NPCs) and transplanted into a contusive SCI injury model survived, differentiated into mature CNS cell types, and led to improved functional recovery (McDonald et al., 1999). Further research showed transplanted ES-NPCs improved spontaneous and evoked pain responses in mice after SCI, suggesting increases in serotonergic fiber regeneration (Hendricks et al., 2006). Surviving transplanted ES-NPCs have been characterized as NG2 positive and express matrix metalloproteinase 9, which is capable of degrading the inhibitory CSPGs to promote axonal outgrowth (Vadivelu et al., 2015).

Similar to NSC transplantation, ES-NPCs have poor survival post transplantation, and the surviving cells typically differentiate into glia with poor neuronal differentiation and maturation. The optimal time for stem cell delivery into the injured rat spinal cord has been established as 1-2 weeks post-injury corresponds to the tail end of the inflammatory phase and beginning of glial scar formation (Ogawa et al., 2002, Okano, 2002, Okano et al., 2003). The high glia differentiation led to the development of more restricted neuronal domains and protocols to increase the purity of motor neuron progenitors prior to transplantation (Erceg et al., 2009, Rossi et al., 2010). High purity human ESC-derived motor neuron progenitors (hMNPs) were transplanted into rat cervical SCIs where they survived, differentiated, and demonstrated site-specific integration distal to the injury site. Of interest, hMNPs near the injury site reverted to a neuronal progenitor state, which suggests that the environmental cues at the injury site limit neuronal maturation.

Ethical concerns surrounding the use of human ESCs has given rise to the interest in human induced pluripotent stem cells (hiPSCs) as a cell source. Recent publications have shown that hiPSCs

differentiated using ESC differentiation protocols for progenitor motor neurons result in phenotypically similar cells that are functional upon transplantation into chick embryos and form functional connections with host tissue (Toma et al., 2015). Transplantation of hiPSC neurospheres into SCI models has resulted in the maturation of astrocytes and neurons with improved angiogenesis, host tissue sparing, and functional recovery (Nori et al., 2011, Kobayashi et al., 2012) (Kobayashi et al 2012, Nori et al 2011). Interest in neural progenitor cells is continually growing and maturing as evidenced by the human ESC-derived NSCs clinical trial that began in 2014 by Neural Stem Cells, inc. (Rockville, MD) and has now entered into Phase II clinical trials that will study the efficacy of NSCs to treat cervical SCI.

One large drawback to the use of ESC-derived neural cell types is the formation of teratomas due to the small percentage of undifferentiated ESCs present in the transplant (Johnson et al., 2010b). To address this problem, the Sakiyama-Elbert lab in collaboration with Dr. David Gottlieb's lab has developed transgenic ESCs that express antibiotic resistance under the progenitor motor neuron marker, Olig2 (McCreedy et al., 2012). This allows for antibiotic selection of ESC-derived pMNs, allowing for transplantation of highly pure populations. Selected ESC-derived pMNs embedded in modified fibrin scaffolds with neurotrophic factors survived and differentiated in a sub-acute rat thoracic SCI model (McCreedy et al., 2014). Most importantly, the transplants were negative for ESC markers and teratomas did not form. The ability to remove the tumorgenic properties of the ESC-derived transplants removes a major concern for clinical applications, especially given their efficacy.

1.4 Biomaterial Scaffolds used for Spinal Cord Injury Therapy

Biomaterial scaffolds are formed from synthetic or natural sources and can provide a structural bridge between injured areas, promote axonal extension and cell migration, and mediate sustained release of molecular therapies directly to the injured site. Although biomaterial scaffolds play a key role in repair following SCI, they are commonly used in conjunction with drug delivery and cell transplantation therapies. Many studies have shown the efficacy of coupling cell transplantation with biomaterial scaffolds because cell transplantation alone typically leads to low survival due to the unfavorable environment. While many synthetic and natural biomaterials have been studied in animal models, only the common natural scaffolds will be reviewed here. Synthetic scaffolds have many advantages including customization and control over mechanical properties, but can be prone to immune responses and are limited by the FDA (Straley et al., 2010). A combination of natural and synthetic scaffolds that incorporate the best of both systems may be a promising therapeutic biomaterial scaffold going forward.

Natural scaffolds provide unique benefits over synthetic scaffolds, including their biocompatibility, high quantity of cell adhesion sites, and approval from the FDA for use as transplant material. Natural scaffolds typically have lower mechanical properties and are limited to applications in soft tissues such as skin, PNS, and CNS; materials for SCI treatment are typically chosen for their cell adhesion sites to promote cell survival and migration (Kim et al., 2014). Three common natural scaffolds reviewed below are agarose, collagen, and fibrin, but research has also focused on alginate, chitosan, and hyaluronic acid with varying results.

Alginate/Chitosan

Alginate has shown some promise when BDNF-expressing fibroblasts were incorporated into the scaffold, but alginate has known cytocompatibilty issues and a high degradation rate, which

make it a less ideal material for SCI treatment (Zimmermann et al., 2001, Tobias et al., 2005, Ashton et al., 2007). Chitosan scaffolds are one of the few biomaterials that demonstrate neuroprotective effects and potential for functional recovery without combination with cell transplantation or drug delivery (Cho et al., 2010). Some research has shown that transplantation of NSCs in chitosan channels resulted in improved regeneration and functional recovery in complete transections of thoracic rat spinal cords (Nomura et al., 2008a, Nomura et al., 2008b, Bozkurt et al., 2010). However, no significant effects were seen when chitosan channels were loaded with anti-myelin inhibitors to remove extrinsic inhibitory factors or cAMP to increase neuronal growth potential (Kim et al., 2011, Guo et al., 2012).

Hyaluronic Acid

Hyaluronic acid scaffolds have several limitations, including limited cell adhesion, weak mechanical properties, and high degradation rates. For use in SCI, hyaluronic acid scaffolds must be modified to incorporate cell adhesion sites into the material and combined with synthetic polymers to improve mechanical properties. For instance, oligodendrocyte progenitor cells were transplanted within hyaluronic scaffolds crosslinked with thiol-functionalized gelatin and poly(ethylene glycol) diacrylate and resulted remyelination of demylinated axons after ethidium bromide treatment (Li et al., 2013).

Agarose

Agarose, collagen, and fibrin are more commonly used for as biomaterial scaffolds in SCI due to their ability to provide cell adhesion, form *in situ* under mild conditions, and high cell transplant potential. Agarose has shown potential to guide cell adhesion and neurite outgrowth *in vitro* (Luo and Shoichet, 2004) and increase axonal regeneration following SCI (Stokols and

Tuszynski, 2006). Templated agarose scaffolds containing autologous BMSCs that ubiquitously express NT-3 showed significant increases in long range axonal growth of transected cervical dorsal column sensory axons (Grous et al., 2013). However, regeneration beyond the scaffold was limited, most likely due to the reactive glial scar, further demonstrating the need to remove inhibition caused from secondary injury.

Collagen

Collagen is a promising natural biomaterial due to the abundance of cell adhesion sites capable of supporting migration, proliferation, and differentiation, and also has mechanical properties similar to most soft tissues (Yoshii et al., 2004). Collagen scaffolds combined with growth factors, BDNF and NT-3, in a dorsal column transection resulted in increased corticospinal tract regeneration and partial functional recovery (East et al., 2010, Han et al., 2010). Transplantation of ESC-NPCs or Schwann cells in collagen scaffolds each led to enhanced remyelination and axon regeneration in rat models of SCI (Hatami et al., 2009, Patel et al., 2010); however, only the transplantation of ESC-derived neural precursors showed improved functional recovery. Recently, the transplantation of OECs within a collagen-based multichannel scaffold resulted in no discernable improvements in motor function or decrease in allodynia following a thoracic hemisection in rats (Deumens et al., 2013). This adds doubt to the efficacy of transplanting OECs or using collagen scaffolds to treat SCI.

Fibrin

Fibrin based scaffolds have been considered one of the best candidates for a minimally invasive scaffold with high therapeutic potential (Itosaka et al., 2009, Kim et al., 2014). Similar to collagen, fibrin has a multitude of cell adhesion sites and can support cell migration, proliferation,

and differentiation. As with most natural scaffolds, fibrin scaffolds are biodegradable and can be injectable or formed *in situ*. The transplantation of fibrin scaffolds with NT-3 growth factor after injury resulted in increased neural fiber density but no significant improvements in functional recovery at 12 weeks (Taylor et al., 2006c). The combination of NT-3 and PDGF growth factors delivered from fibrin scaffolds transplanted in a sub-acute model (two weeks after the initial injury) led to increased neural fiber density, promoted neuronal extensions across the lesion barrier, and decreased development of the glial scar (Johnson et al., 2010b) .

Additional studies transplanted ESC-derived NPCs in fibrin scaffolds with NT-3 and PDGF growth factors, which caused improved cell survival and proliferation, although sustained release of the growth factors increased tumor formation at the site of injury (Johnson et al., 2010c). More recently, fibrin scaffolds containing growth factor cocktails and NSCs were transplanted in complete thoracic transection in rats, which resulted in axonal projections through the transection and back into the host tissue and showed significant locomotor recovery. These results were extremely promising, but replication studies failed to discern any increases in functional recovery compared to control groups, measured limited host axons growing into the transplant site, and saw ectopic masses of transplanted NSCs long distances away from the transplantation site including the brain (Sharp et al., 2014, Steward et al., 2014).

NSC mediated tumor and ectopic mass formation truly highlights the need for improved cell transplantation models when using NSCs. To this end, high purity progenitor domains from ESC-derived pMNs were purified using antibiotic resistance expressed under the Olig2 promoter, which is expressed by cells in the pMN domain. Transplantation of the selectable pMNs within fibrin scaffolds containing NT-3 and PDGF led to significant decreases in the tumorgenic potential of the transplanted cells (McCreedy et al., 2014). Fibrin scaffolds have shown promising results as cell

transplantation and drug delivery vehicles and continue to at the fore front for materials used to treat SCI.

1.5 Concluding Remarks

The field has made tremendous strides in understanding the pathology of and mechanisms behind spinal cord injury since the ground-breaking experiments performed by Aguayo in 1980. Over 35 years, the acute, sub-acute, and long term effects of SCI have been teased out through the development of diverse animal injury models and the amazingly complex adult CNS, once considered "something fixed, ended, immutable," (Ramon y Cajal, 1928) is now viewed as plastic, tailorable, and fixable. Most of the major players and obstacles are known, and a plethora of therapeutic strategies are being researched with several making progress in clinical trials. Although much work must still be done, it is clear that the future of SCI treatment is promising and it is important to remember that modest increases in functional recovery can lead to robust improvements in the quality of life of SCI patients.

In this introduction I have tried to highlight the importance of using combination strategies to overcome multiple obstacles found after SCI. Single therapeutic interventions have given way to combination strategies that synergistically work together to improve recovery. Molecular based therapies that deliver exogenous growth factors, anti-inhibitory molecules, or axonal growth promoters have been combined with cellular transplantation therapies using genetic engineering or biomaterials-based drug delivery systems. The combination of cell transplantation and biomaterial scaffolds is of utmost importance due to the increased survivability of the transplanted cells. The biomaterial scaffold provides a growth substrate for cell migration and neuronal extension into and across the injury site while transplanted cells can secrete a wide swath of signaling and neurotrophic molecules that aid recovery.

The Sakiyama-Elbert lab has consistently been working toward incorporating new factors into already multifactorial therapeutic interventions. The most recent iteration of the tissue engineered system transplanted high purity pMNs within modified fibrin scaffolds containing NT-3 and PDGF. The fibrin scaffolds are modified with a bi-domain peptide containing an ATIII binding sequence that attaches the peptide to the fibrin scaffold and a heparin binding domain, which can bind to heparin. When heparin is included in the gelation solution, the heparin binding domains located in the tertiary structures of NT-3 and PDGF retain the neurotrophic factors and cause slow release from the scaffold. The pMNs used were developed in collaboration with Dr. David Gottlieb; the transgenic ESC line (P-Olig2) removes the tumorgenic potential of ESC-derived cells by expressing the puromyocin resistance enzyme, puromyocin *N*-acetyl-transferase, when regulatory elements of the Olig2 protein are expressed. Selected cells were shown to survive and differentiate into astrocytes, oligodendrocytes, and neurons in a sub-acute SCI model (McCreedy et al., 2014). The combination therapy is capable of repopulating lost cells, secreting beneficial neurotrophic factors, and providing a pro-growth substrate for cell migration and axonal extension. However, this system does not currently provide a mechanism that limits inhibition due to the formation of the glial scar and remaining myelin debris. Thus, the focus of this thesis is on developing, characterizing, and incorporating anti-inhibitory drug delivery systems into the previously established tissue engineered scaffolds.

The two main inhibitory factors around the injury site are CSPGs and MAIs, which were discussed in section 1.2.2. The first aim of this work focused on removing the inhibition seen from the two inhibitory molecules using ChABC against CSPGs and NEP1-40 against MAIs. The delivery of ChABC and NEP1-40 has previously been achieved through microinjections and invasive intrathecal pumps, but we wanted to incorporate the anti-inhibitory molecules into the fibrin

scaffold system. Therefore, the first aim also developed drug delivery systems capable of loading and releasing active ChABC and NEP1-40.

The second aim built upon the first by transitioning the developed anti-inhibitory drug delivery systems into an animal model of SCI. To evaluate the efficacy of the developed drug delivery systems, they were incorporated into fibrin scaffolds and transplanted into an acute thoracic SCI model using adult rats. The transplants were placed immediately after injury and the spinal cords were harvested two weeks post-transplantation. The ability of the drug delivery systems to limit the inhibitory effects of CSPGs and improve axonal extension was measured. A sub-acute injury model was performed with the full combination therapy including neurotrophic factors, anti-inhibitory molecules, and cell transplantation within the modified fibrin scaffolds.

Chapter 2

Developed Drug Delivery Systems Overcome Inhibition from the Glial Scar

2.1 Abstract

Myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) are major contributors to axon growth inhibition following spinal cord injury and limit functional recovery. The NEP1-40 peptide competitively binds the Nogo receptor and partially blocks inhibition from MAIs, while chondroitinase ABC (ChABC) enzymatically digests CSPGs, which are upregulated at the site of injury. *In vitro* studies showed that the combination of ChABC and NEP1-40 increased neurite extension compared to either treatment alone when dissociated embryonic dorsal root ganglia were seeded onto inhibitory substrates containing both MAIs and CSPGs. Furthermore, the ability to provide sustained delivery of biologically active ChABC and NEP1-40 from biomaterial scaffolds was achieved by loading ChABC into lipid microtubes and NEP1-40 into poly (lactic-co-glycolic acid) (PLGA) microspheres, obviating the need for invasive intrathecal pumps or catheters. Fibrin scaffolds embedded with the drug delivery systems (PLGA microspheres and lipid microtubes) were capable of releasing active ChABC for up to one week and active NEP1-40 for over two weeks in vitro. In addition, the loaded drug delivery systems in fibrin scaffolds decreased CSPG deposition and development of a glial scar, while also increasing axon growth after spinal cord injury *in vivo*. Therefore, the sustained, local delivery of ChABC and NEP1-40 within the

injured spinal cord may block both myelin and CSPG-associated inhibition and allow for improved axon growth.

2.2 Introduction

Spinal cord injury (SCI) is a major medical problem affecting ~320,000 Americans, with 12,000 new cases occurring annually (NSCIS, 2012). SCI typically results in partial or complete loss of function below the initial site of injury, leaving patients without the ability to perform basic daily activities. Loss of function occurs by disrupting signal transduction of ascending and descending neuronal tracts. After the initial contusion or compression injury, a secondary injury leading to the formation of a glial scar causes increased cell death at the site of injury, as well as the upregulation of inhibitory factors that limit recovery (Schwab and Bartholdi, 1996). Current research focuses on reforming functional synapses between severed tracts by increasing the intrinsic growth capacity of neurons, removing the extrinsic barriers to regeneration from inhibitory cues, or replacing lost cells by transplantation (Kadoya et al., 2009, Mothe and Tator, 2012, Sharma et al., 2012).

Therapies for SCI are trending toward multifunctional systems that combine single therapies to overcome several inhibitory obstacles and further enhance functional recovery (Kadoya et al., 2009, van den Brand et al., 2012, Zhao et al., 2013). The use of biomaterial scaffolds allows for the development of multifunctional systems by providing a growth substrate for host and transplanted cells, delivery of growth factors that promote cell survival, and delivery of anti-inhibitory molecules to increase migration and axon extension following injury. The transplantation of neural progenitors, olfactory ensheathing cells, Schwann cells, and other cell types have shown improved benefits when transplanted within natural or synthetic biomaterial scaffolds (Fortun et al., 2009, Sahni and Kessler,

2010, Hernandez et al., 2011, Tetzlaff et al., 2011). Previous research in our lab has shown the benefits of transplanting embryonic stem cell-derived neural progenitor cells within a fibrin-based scaffold capable of sustained delivery of neurotrophic factors (Johnson et al., 2010a). Transplantation of neural progenitor cells coupled with the delivery of neurotrophic factors promoted recovery by repopulating the cystic cavity in the lesion, increasing transplanted and host cell survival, and increasing the growth potential of neurons. However, further improvements to the scaffold include the addition of drug delivery systems that release anti-inhibitory molecules, which reduce the extrinsic barriers within the glial scar that block axonal extension.

The formation of a glial scar greatly limits axonal regeneration into and across the injury site. The glial scar is generated by reactive astrocytes that extend processes to form a physical and chemical barrier to axonal extension (Sharma et al., 2012). Key chemical inhibitors to axonal extension are chondroitin sulfate proteoglycans (CSPGs) and myelin-associated inhibitors (MAIs). CSPGs and MAIs have been studied extensively due to their ability to destabilize the axonal growth cone by initiating the Rho/ROCK signaling cascade (Iaci et al., 2007, Schwab, 2010). The deposition of CSPGs is significantly upregulated after SCI for up to 8 weeks, with most deposition occurring within 2 weeks after the primary injury (Jones et al., 2003). Chondroitinase ABC (ChABC) has been extensively studied and shown to limit the effects of CSPGs by cleaving inhibitory sugar chains, leaving the core protein and stub carbohydrates (Bradbury et al., 2002a, Chau et al., 2004, Lee et al., 2013).

Intrathecal delivery of ChABC into severely injured spinal cords for 10 days post-injury decreased the level of deposited CSPGs, increased axonal extension, and significantly improved functional recovery. Research has shown that the use of an implantable intrathecal pump to deliver ChABC every other day for 2 or 4 weeks in cat hemisection models led to differences in functional

recovery and that longer treatment times increased the number of rubrospinal tract neurons below the lesion site (Mondello et al., 2015). The use of the implantable injection system verifies the need of prolonged delivery of ChABC but requires implantation of an invasive pump system for 4 weeks. The need for invasive pumps or microinjections has been removed by delivery of the highly labile ChABC enzyme through natural and synthetic drug delivery systems (Huang et al., 2011, Liu et al., 2012, Rossi et al., 2012, Pakulska et al., 2013, Bartus et al., 2014). In addition, combination therapies with ChABC have also shown promise, but limited research has been performed with both ChABC drug delivery systems coupled with other promising therapies (Liu et al., 2012, Zhao et al., 2013, Kanno et al., 2014). In this study, we developed a combination therapy of dual anti-inhibitory molecules to improve upon single treatment options that utilizes natural and synthetic drug delivery systems to remove the need for intrathecal pumps and microinjections.

In addition, several techniques have tried to limit the effects of MAIs following SCI by blocking the interaction of the myelin glycoprotein, Nogo-A with its receptor, NgR1, found on axons (GrandPre et al., 2000, GrandPre et al., 2002, Liebscher et al., 2005b, Maier et al., 2009, Huebner et al., 2011). Specifically, the NEP1-40 peptide has been shown to limit the effect of MAIs through competitive inhibition of Nogo-A binding to NgR1. Intrathecal delivery of NEP1-40 for 4 weeks post-injury led to increased axonal extension and functional recovery after thoracic SCI in rats (GrandPre et al., 2000, GrandPre et al., 2002, Cao et al., 2008). The NEP1-40 peptide has also been administered through intraperitoneal injection and subcutaneous osmotic pumps in mouse SCI with both showing improved axonal sprouting rostral to SCI (Li and Strittmatter, 2003). Furthermore, transplantation of hybridoma cells expressing a monoclonal antibody, IN-1, that binds to Nogo and blocks myelin-associated inhibitor signaling also showed improved recovery over cells expressing a control antibody (Merkler et al., 2001). These demonstrate that the duration and therapeutic window for treatment with anti-myelin inhibitors is important to increase axon sprouting and functional

recovery; extended treatment durations chronically suppress MAIs and delayed therapies of at least 1 week showed no difference to immediate treatments (Merkler et al., 2001, Li and Strittmatter, 2003).

While ChABC and NEP1-40 have been found effective individually, the combined delivery of ChABC and anti-myelin inhibitors using cannulas and intrathecal pumps, along with treadmill training, significantly increased functional recovery compared to either treatment alone (Zhao et al., 2013). Therefore we hypothesized that it would be beneficial to build upon previously published work by developing a drug delivery system that releases multiple anti-inhibitory molecules and can be combined with biomaterial-based cell transplantation therapies. Our drug delivery systems are introduced simultaneously but have varying release profiles. Thus the delivery of individual anti-inhibitory molecules can be tuned independently. In this study, we developed drug delivery vehicles capable of providing sustained delivery of both ChABC and NEP1-40 over one week *in vitro*. Also, proof of concept studies were performed by incorporating the drug delivery vehicles into fibrin scaffolds and implanting into injured rat spinal cords. The dual delivery of ChABC and NEP1-40 limited the development of the glial scar and increased the number of axons found around the injury site.

2.3 Materials and Methods

2.3.1 Myelin Purification

Myelin was purified using previously published protocols (Norton and Poduslo, 1973). Briefly, adult rat brains were harvested and placed into 0.32 M ice cold sucrose, homogenated, and overlaid on top of equal volumes of 0.85 M sucrose in ultracentrifuge tubes. The solution was centrifuged at 75,000 g for 30 minutes. The crude white myelin layer at the interphase was collected and added to ice cold sterile ultrapure water and centrifuged at 75,000 g for 15 minutes. The supernatant was discarded and ultrapure water was added to the pellet and centrifuged at 12,000 g for 10 minutes. The supernatant is discarded, ultrapure water added to the pellet, and then centrifuged at 12, 000g for 10 minutes again. The supernatant was discarded again and the pellet resuspended in 1x sterile phosphate buffered saline, pH 7.4. Protein concentration was determined using a protein quantification assay kit (Bio-Rad Laboratories, Hercules, CA) and polyacrylamide gel electrophoresis was used to compare the purified myelin protein's molecular weights to previously published results for rat CNS.

2.3.2 Inhibitory Spot Assays

Chick dorsal root ganglia (DRG) neurite outgrowth assays were used similar to previous publications (GrandPre et al., 2002). Briefly, dissociated embryonic day 8 chick DRG cells were cultured on purified myelin, CSPG (EMD Millipore, Billerica, MA), or combination purified myelin and CSPG spots. Prior to plating, 24-well tissue culture plates were coated with poly-L ornithine (Sigma, St. Louis, MO) for one hour at 37°C, then washed 3 times with water, and dried overnight. After drying, 1 μL spots of inhibitory substrate (87.5 μg/mL of myelin or 10 μg/mL of CSPG) in phosphate buffered saline (PBS, pH7.4) was placed onto the coated wells and dried overnight.

Dissociated DRGs were plated at 100,000 cells/well, fixed with 4% paraformaldehyde (Sigma) after 6 hours, and stained for neurofilament (NF, Dev. Studies Iowa Hybridoma Bank, Iowa City, IA, 1:200). Stained neurites were imaged using a 20x objective on an Olympus IX70 (Olympus, Center Valley, PA) inverted microscope with an Optronics MICROfire camera (Optronics, Muskogee, OK) and neurite lengths were measured using ImageJ software with over 100 neurites measured per group. Culture medium was 1:1 DFK5:Neurobasal with B27 (Life Technologies, Carlsbad, CA) and varying concentrations of ChABC or NEP1-40. DFK5 media consisted of DMEM:F12 base media (Life Technologies) supplemented with 5% knockout serum replacement (Life Technologies), 50 μg/ml apo-transferrin (Sigma), 50 μM non-essential amino acids (Life Technologies), 5 μg/ml insulin (Sigma), 30 nM sodium selenite (Sigma), 100 μM β-mercaptoethanol (Life Technologies), 5 μM thymidine, and 15 μM of the following nucleosides: adenosine, cytosine, guanosine, and uridine (EMD Millipore).

2.3.3 Formation of Poly(lactic-co-glycolic acid) (PLGA) Microspheres

PLGA microspheres, with a 50:50 lactic acid to glycolic acid ratio, were fabricated using a water in oil in water double emulsion solvent evaporation technique (Kim et al., 2008b). PLGA (10% w/v, intrinsic vis. = 0.15-0.3 dL/g, Absorbable Polymers Inc., Pelham, NJ) was dissolved in 2 mL dichloromethane (DCM). 100 μL of 10 mg/mL NEP1-40 (Mw = 4627 Da, Sigma) was added to the PLGA/DCM solution and sonicated for 10 seconds (Microson, Misonix Inc.), added to 25 mL of water with 1% w/v poly(vinyl alcohol) and 10% w/v NaCl and homogenized. The resulting emulsion was poured into 250 mL of water with 0.1% w/v poly(vinyl alcohol) and 10% w/v NaCl, then magnetically stirred for 3 hours, washed with water, frozen overnight at -80°C and lyophilized. PLGA microspheres were imaged using a scanning electron microscope (Nova NanoSEM 230, FEI) after gold sputter coating for 45 seconds. Diameters from individual microspheres were measured

using ImageJ software and 100 microsphere diameters were averaged per batch. Loading efficiency of PLGA microspheres was measured using previously established protocols (Lam et al., 2000, Kang and Singh, 2001, Gutierro et al., 2002). Specifically, microspheres were fully degraded with 0.5 M NaOH to release the loaded molecules. An equal volume of 10 mM phosphate buffered saline was added and the solution pH was adjusted to 7 using HCl. The amount of fluorescently loaded molecules was measured using a SpectraMax M2e fluorimeter (Molecular Devices, Sunnyvale, CA).

2.3.4 ChABC Thermostability Assay

The enzymatic activity of ChABC in phosphate buffered saline, pH 7.4 containing 1 M trehalose was measured over a four week period by monitoring the formation of unsaturated disaccharides by degrading the chondroitin sulfate over time (Sigma enzymatic assay – EC 4.2.2.4 per manufacturer instructions). To perform the study, 1 U/mL of ChABC was dissolved in PBS with or without 1 M trehalose added to the solution. The activity of the solution was measured immediately after dissolving and at 2 days, 14 days, and 28 days. The amount of activity remaining at the later time points was compared to the original amount of activity to determine the percent of ChABC that remained active.

2.3.5 Formation of Lipid Microtubes

Lipid microtubes were formed similar to previous work (Meilander et al., 2001). 1,2-bis(tricosa-10,12-diynoyl)-sn-3-phosphocholine (DC8,9PC) lipids (Avanti Polar Lipids, Alabaster, AL) were dissolved at 55°C in 70% ethanol at 1 mg/mL and placed into a temperature controlled water bath (Haake A10, ThermoScientific, Asheville, NC). The total volume per batch was 5 mL. The temperature was decreased from 55°C to 25°C at a rate of 2.5°C/min. The lipid tubes were then stored at room temperature in the dark for one week. Trehalose (EMD Millipore) was added to a final concentration of 50 mM, the solution was centrifuged (1200 rcf, 5 minutes) to pellet the lipid

microtubes, and 4 mL of solution was removed. The concentrated (5 mg/mL) microtube solution was lyophilized overnight to dry and stored at -20°C until further use.

2.3.6 Fluorescein isothiocyanate (FITC) Conjugation to NEP1-40 and ChABC

FITC was covalently conjugated to NEP1-40 and ChABC (Sigma). To fluorescently label the molecules, a solution of 2 mg/mL ChABC or 2 mg/mL NEP1-40 was dissolved in 0.1 M sodium carbonate buffer, pH 9. FITC (Sigma) was freshly dissolved in dimethyl sulfoxide (DMSO) at 1 mg/mL, and 50 μL of the FITC solution was then slowly added to the ChABC/NEP1-40 solution in 5 μL aliquots while continually stirring. The solution was allowed to react for 8 hours at 4°C and protected from light. Ammonium chloride was added to a final concentration of 50 mM and incubated for 2 hours to stop the reaction. The finished reaction was then dialyzed against phosphate buffered saline, pH 7.4, to remove unconjugated FITC. The molecular weight cutoff for the ChABC dialysis cassette was 10 kD and the cutoff for the NEP1-40 dialysis cassette was 2 kD. Following dialysis, the conjugates were stored at 4oC. The ratio of fluorescein to ChABC/NEP1-40 was determined by measuring the absorbance at 495 nm and 280 nm. Electrophoresis with polyacrylamide gels was also used to verify the overlap of fluorescent ChABC protein bands with Coomassie Blue stained bands.

2.3.7 Release profiles of FITC Conjugated NEP1-40 and ChABC

The release profiles of fluorescently labeled NEP1-40 (F-NEP) loaded in PLGA microspheres and fluorescently labeled ChABC (F-ChABC) loaded in lipid microtubes was measured by embedding the microparticles into fibrin scaffolds. Fibrin scaffolds with total volume of 200 μL were prepared by mixing 10 mg/ml fibrinogen, 2.5 mM CaCl2 (Sigma), 2 NIH units/mL thrombin (Sigma), and 30 mg of FITC-NEP1-40 loaded PLGA microspheres or 5 mg of FITC-ChABC loaded microtubes. Scaffolds were allowed to polymerize for one hour at 37°C in a 48 well

plate, then 200 μL of Tris buffered saline was placed on top. The saline solution was removed at 1, 3, 5, 7, 10, and 14 days after scaffold formation, and the fluorescence measured using a fluorimeter. Following the last time point, the fibrin scaffolds and PLGA microspheres were degraded using 1 M NaOH overnight at 37°C, then an equal volume of 10 mM Tris buffered saline was added and the pH was adjusted to 7 using HCl to measure the amount of fluorescent molecules remaining in the scaffold.

2.3.8 Active release of NEP1-40

PLGA microspheres loaded with 40 mg/mL NEP1-40 were incorporated into 10 mg/mL fibrin (EMD Millipore) scaffolds and allowed to release NEP1-40 into cell culture media. The media was removed and replaced on days 5, 12, and 20. The removed media, now containing released NEP1-40, was used as the culture media for inhibitory myelin spot assays. Cell culture media was 1:1 DFK5:Neurobasal with B27 supplement. Control experiments were performed with empty PLGA microspheres.

2.3.9 Active release of ChABC

Lipid microtubes loaded with 500 mU/mL ChABC (Sigma) dissolved in 1 M trehalose were encapsulated into 10 mg/mL fibrin scaffolds and allowed to release ChABC into 1 M trehalose solution. The trehalose solution was tested for enzymatic activity by measuring the formation of unsaturated disaccharides by degrading the chondroitin sulfate over time (Sigma enzymatic assay – EC 4.2.2.4 per manufacturer instructions). Control experiments were performed with lipid microtubes loaded with 1 M trehalose and ChABC directly added to the fibrin scaffold formation solution.

2.3.9 Statistical Analysis

Spot assay data was analyzed by ANOVA followed by Scheffe's post-hoc test with a significance criterion of 95%. Statistical significance for in vivo studies was determined by the Fisher's Least Statistical Difference post-hoc test with a significance criterion of 95%.

2.4 Results

2.4.1 Combination ChABC and NEP1-40 Improve Neurite Extension on Inhibitory Spots

Spot assays were used to study the effect of culturing primary neurons on inhibitory substrates of myelin, CSPGs, or myelin and CSPGs combined (Figure 2.1). When E8 chick DRG explant cultures were plated onto inhibitory spots, neurite outgrowth was significantly inhibited compared to neurons plated outside of the inhibitory spots. To determine the percent of inhibition caused by plating on inhibitory spots, the average neurite length of cells on the inhibitory substrates was divided by the average neurite length of cells plated outside the inhibitory substrates on poly-L ornithine. Neurons plated outside of the inhibitory spots had an average neurite length over 50 μm compared to neurites inside inhibitory spots, which had average lengths of less than 20 μm.

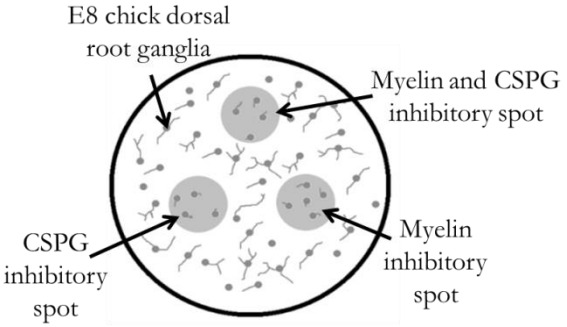

Figure 2.1: Representative image of a spot assay. Dots represent inhibitory substrates with neurons plated and extending neurites inside and outside the spots.

Furthermore, a significant increase in neurite length of cells plated onto inhibitory CSPG spots was measured when increasing amounts of ChABC was added to the culture media (Figure 2.2). The group with 100 mU/mL ChABC added had the greatest amount of neurite growth compared to 0 mU/mL, 1 mU/mL, and 10 mU/mL but no treatment increased neurite length back to uninhibited levels.

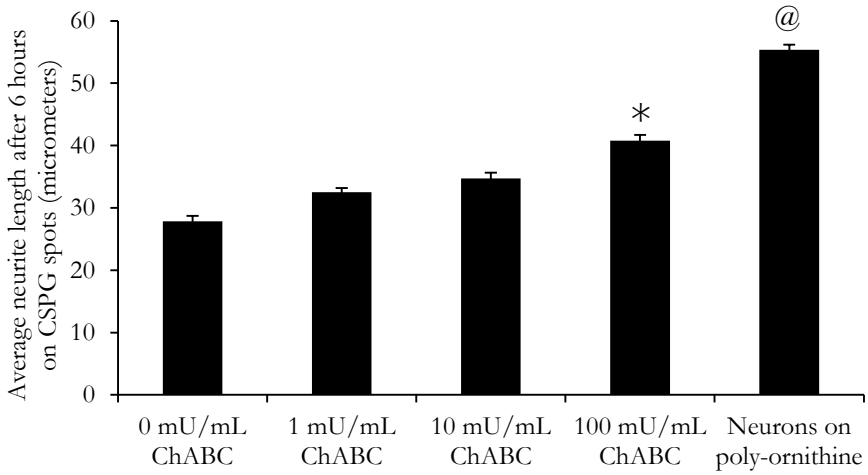

Figure 2.2: A**verage neurite length of dissociated DRG neurons on CSPG spots 6 hours after plating.** The positive control is neurons plated outside of the inhibitory spots. n > 100. Error bars are standard error of the mean. * denotes significance from 0 mU/mL ($p<0.05$). # denotes significance from 10 mU/mL ($p<0.05$). @ denotes significance from 100 mU/mL ($p<0.05$).

The same trend was seen when culturing cells on myelin inhibitory spots with increasing concentrations of NEP1-40 added to the cell media (Figure 2.3). The highest concentration of NEP1-40 tested was 500 nM which had significantly increased neurite extension compared to 0 nM, 10 nM, and 100 nM concentrations of NEP1-40 but again the increase in neurite extension did not fully recover to uninhibited levels.

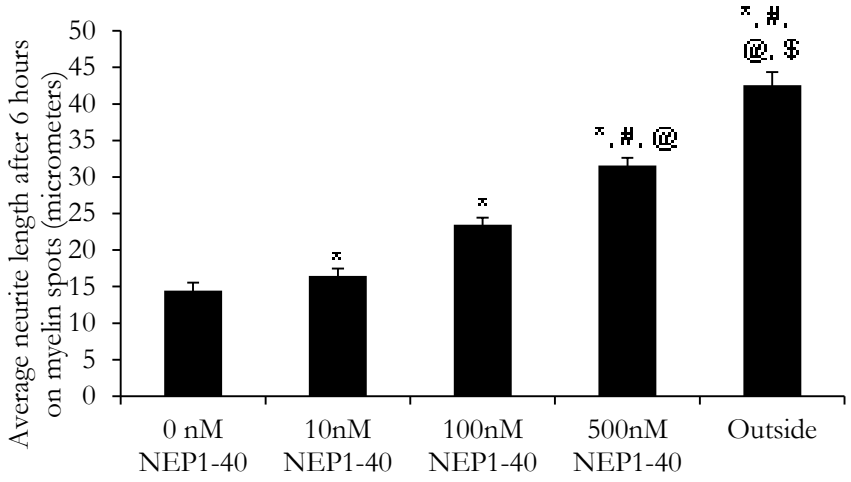

Figure 2.3: Average neurite length of dissociated DRG neurons on myelin spots. The positive control is neurons plated outside of the inhibitory spots. n > 400. Error bars are standard error of the mean. * denotes significance from 0 nM ($p<0.05$). # denotes significance from 10 nM ($p<0.05$). @ denotes significance from 100 nM ($p<0.05$). $ denotes significance from 500 nM ($p<0.05$).

We next studied how the anti-inhibitory molecules affect neurite growth on varying inhibitory spots. The ability of NEP1-40 or ChABC to block myelin inhibition was studied by adding 500 nM of NEP1-40 or 100 mU/mL ChABC to the cell culture media (Figure 2.4). The presence of NEP1-40 attenuates neurite outgrowth inhibition on myelin spots while the addition of ChABC did not significantly affect extension. The presence of NEP1-40 led to an average neurite length of 28.3±1.9 μm, which corresponds to 54% outgrowth compared to the positive control group plated outside the inhibitory spot. The group with no NEP1-40 added to the cell culture media averaged 18.2±0.9 μm or 35% outgrowth compared to the positive control group. The combined addition of NEP1-40 and ChABC had similar effects compared to adding NEP1-40 alone for culture on myelin spots. Therefore, the addition of ChABC to the cell culture media did not affect neurite extension on myelin inhibitory spots but NEP1-40 significantly increased neurite extension.

Similar types of results were seen when culturing dissociated DRGs on CSPG spots. In this case, media containing ChABC significantly increased neurite extension compared to media containing no anti-inhibitory factors or containing NEP1-40 alone (Figure 2.4). The presence of ChABC led to an average neurite length of 27.7±1.2 μm compared to the group without ChABC added which averaged of 17.5±1.1 μm. The presence of ChABC increased the percent outgrowth compared to the positive control to 53%, up from 34% outgrowth in the group without ChABC.

To study the effects of neurite extension in the presence of both ChABC and NEP1-40, inhibitory spots containing both CSPGs and MAIs were used. Spots containing both myelin and CSPGs were capable of providing robust inhibition to neurite outgrowth with an average neurite length of 16.2±0.5 μm when no anti-inhibitory drugs were added to the media (Figure 2.4). The addition of NEP1-40 or ChABC caused a significant increase in average neurite length (19.9±0.4 μm and 20.9±0.6 μm, respectively). Interestingly, the combined addition of NEP1-40 and ChABC in the cell culture media led to a statistically significant increase in neurite length, of 26.1±0.8 μm, compared to either of the anti-inhibitory factors alone. The addition of both anti-inhibitory factors caused a 50% outgrowth compared to the positive control group, while a 38% outgrowth was measured for NEP1-40 addition and 40% outgrowth for ChABC addition. Therefore, the combined effects of NEP1-40 and ChABC treatment may lead to improved axonal extension after SCI.

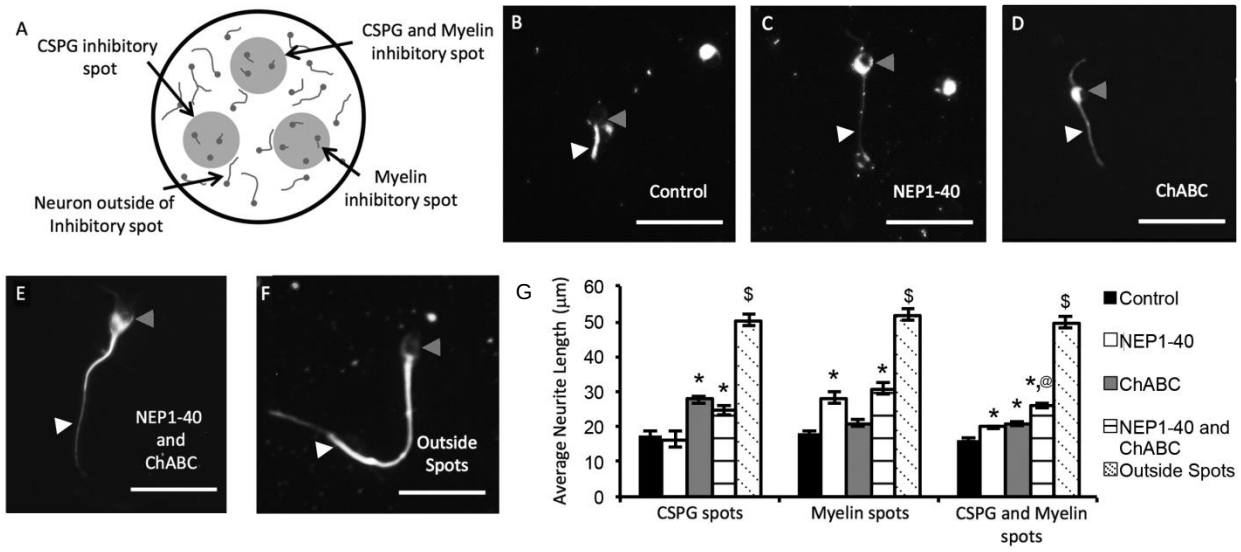

Figure 2.4: Dissociated embryonic chick DRG cultured on myelin and CSPG inhibitory spots and treated with ChABC and NEP1-40. (A) Schematic representation of dissociated dorsal root ganglia neurons cultured on inhibitory spots. Three 1 μL spots of CSPGs, myelin, or CSPGs and myelin were placed onto poly-*L*-ornithine coated 24 well plates. ChABC, NEP1-40, or ChABC and NEP1-40 were directly added to the cell media. Neurons that plate outside of the inhibitory spots were used to determine the average neurite extension of uninhibited neurons. **(B-E)** Representative images of dissociated DRG neurons cultured on combined CSPG and myelin inhibitory spots. White arrowheads indicate neurites and gray arrowheads indicate neuronal cell bodies **(B)** No ChABC or NEP1-40 treatment **(C)** NEP1-40 **(D)** ChABC **(E)** ChABC and NEP1-40 and **(F)** Uninhibited neurons plated outside inhibitory spots. **(G)** The average neurite length was measured for all neurites. Error bars are standard error of the mean. * denotes significance from control ($p<0.05$). @ denotes significance versus NEP1-40 or ChABC treatment ($p<0.05$). $ denotes significance from all other groups. Scale bars are 50 μm.

2.4.2 Incorporation of NEP1-40 into PLGA Microspheres Enables the Sustained Release of Active NEP1-40

Delivery of NEP1-40 was achieved by encapsulation into PLGA microspheres (Figure 2.5). PLGA microspheres provide tunable release rates by controlling the ratio of lactic acid to glycolic acid, the intrinsic viscosity of the polymer, or by varying the formation conditions (Pean et al., 1998, Yang et al., 2000b, Takai et al., 2011). The microspheres were loaded with FITC conjugated NEP1-40 (F-NEP) and embedded into fibrin scaffolds to directly measure the release rate (Figure 2.6).

PLGA microspheres had a loading efficiency of 42.1±9.8% and an average diameter of 9.1±4.2 μm. When F-NEP1-40 was loaded directly into fibrin scaffolds over 80% of the fluorescent molecules were released in 5 days with a large burst release of 41% at day 1. In contrast, loading of F-NEP1-40 into PLGA microspheres lowered the initial burst release to 7%, and provided sustained release with 31% of loaded F-NEP1-40 released at day 5. The remaining F-NEP1-40 was recovered by degrading the fibrin scaffolds and PLGA microspheres using 0.5 M NaOH, adjusting the pH to 7, and then measuring the fluorescence. This gave us the total amount of NEP1-40 remaining within the fibrin scaffolds and PLGA microspheres. These amounts were within 10% of the initial loading dose. The release profile for loading into PLGA microspheres appears to have a first-order release up to 14 days.

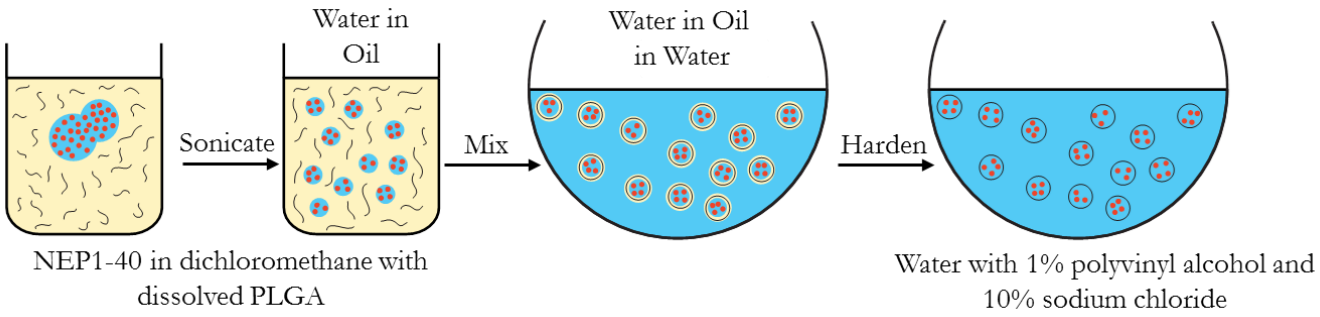

Figure 2.5: Representative image of PLGA microsphere formation. NEP1-40 is dissolved in water and added to a solution of dichloromethane with dissolved PLGA. The solution is mixed with a probe sonicator to form droplets of water. The water in oil solution is then added to a large amount of water with polyvinyl alcohol and sodium chloride. The water in oil in water solution is then mixed for 4 hours to allow evaporation of the dichloromethane and hardening of the microspheres.

The ability to release NEP1-40 from PLGA microspheres is only useful if the peptide remains active after formation of the microspheres, incorporation into fibrin scaffolds, and upon release from the scaffolds. The activity of the released NEP1-40 was analyzed by loading NEP1-40 into PLGA microspheres, embedding the microspheres into fibrin scaffolds, and allowing the

microspheres to release the loaded NEP1-40 into cell culture media. At days 5, 12, and 20 the cell culture media was collected and used for inhibitory spot assays with myelin spots. Dissociated DRGs that were plated onto the myelin spots showed a significant increase in average neurite length with media containing released NEP1-40 compared to control media which did not contain released NEP1-40 at every time point studied (Figure 2.7). Treated media with released NEP1-40 from day 1 through day 5 increased the percent neurite outgrowth compared to outside positive controls to 56%, while media from day 5 through day 12 showed 57% outgrowth compared to positive controls, and media from day 12 through day 20 showed 60% neurite outgrowth. Thus, PLGA microspheres are capable of providing sustained release of NEP1-40, and the released peptide maintains its bioactivity for up to 20 days in vitro.

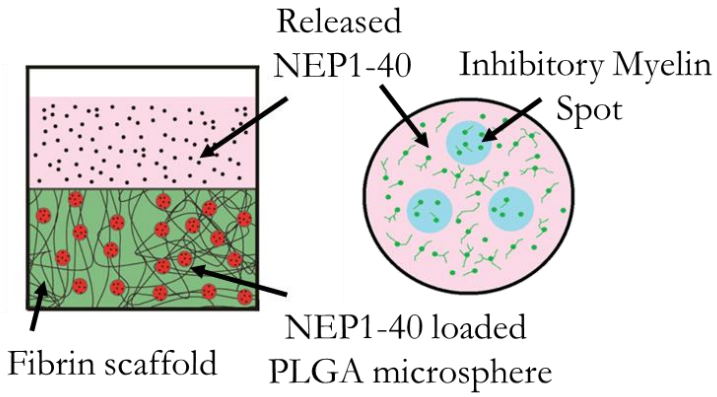

Figure 2.6: Representative depiction of fibrin scaffolds with embedded NEP1-40 PLGA microsphere drug delivery systems. The released NEP1-40 will diffuse into the cell media on top of the fibrin scaffold which is collected and used as media for cells plated on inhibitory myelin spot assays.

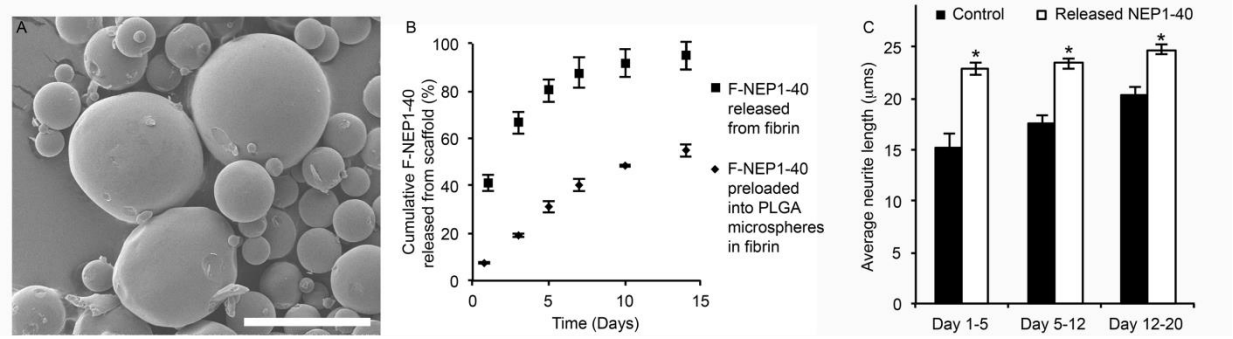

Figure 2.7: Incorporation of NEP1-40 into PLGA microspheres and fibrin scaffolds. (A) Scanning electron micrograph of NEP1-40-loaded PLGA microspheres. Average microsphere diameter measured 9.1±4.2 μm. Scale bar is 30 μm. **(B)** Release profile of F-NEP from PLGA microspheres encapsulated in fibrin scaffolds or from F-NEP in fibrin scaffolds alone. Loading efficiency of microspheres was 42.1±9.8%. **(C)** Dissociated DRGs plated onto inhibitory myelin spots suggest that media containing released NEP1-40 shows significantly less inhibition of neurite extension compared to control (media without NEP1-40) on the same day. Thus, the NEP1-40 loaded into PLGA microspheres within fibrin scaffolds maintains bioactivity after being released into the cell culture media. Error bars denote standard error of the mean. * denotes significance from control group on the same day ($p < 0.05$).

2.4.3 Incorporation of thermostabilized ChABC into Lipid Microtubes Allows Sustained Active Release of ChABC

Delivery of thermostabilized ChABC enzyme was achieved by stabilization with trehalose and loading into lipid microtubes (Meilander et al., 2001). Thermostabilization of ChABC is required prior to loading into lipid microtubes due to the enzyme losing over 70% enzymatic activity in 2 days compared to stabilization with trehalose which lost less that 50% activity. In addition, enzymatic activity was still measured up to 2 weeks when stabilized with trehalose (Figure2.8).

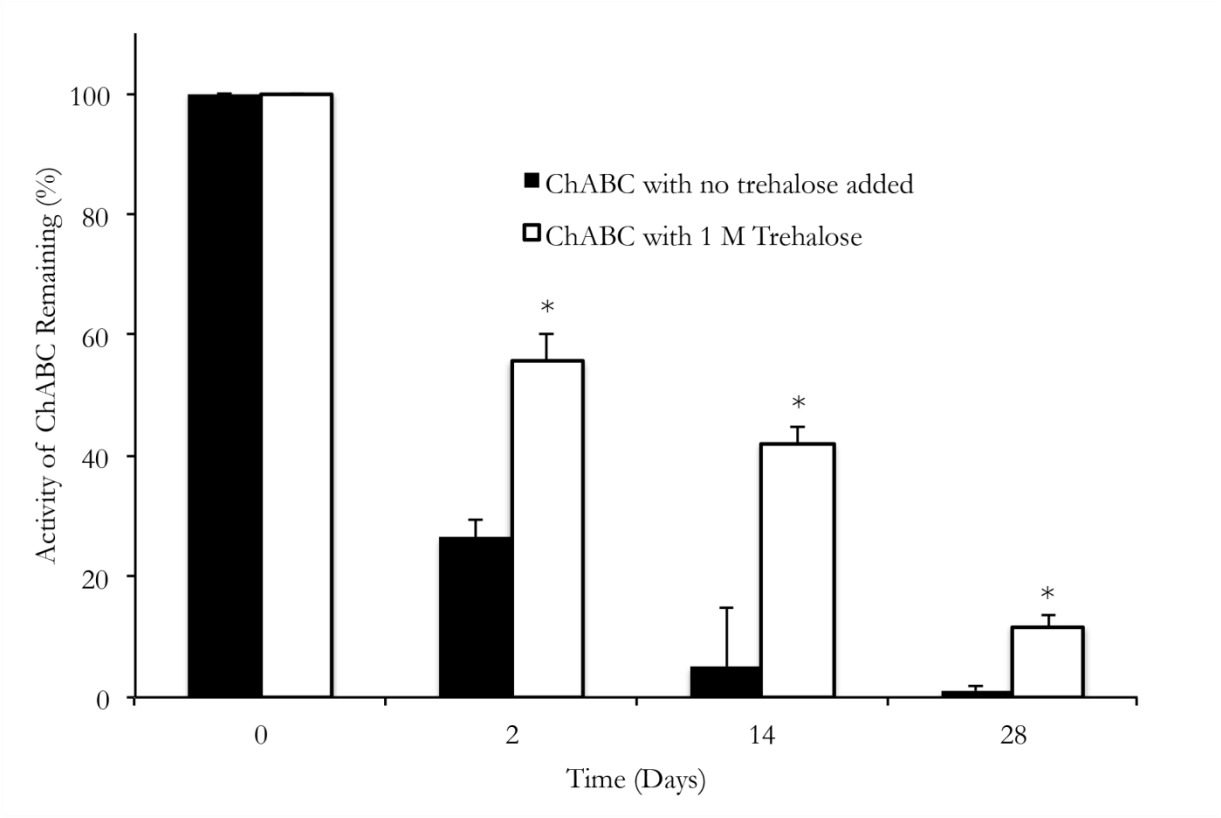

Figure 2.8: Thermostabilization of ChABC with trehalose. Enzymatic activity assay measuring the amount of cleaved chondroitin sulfates in solution showed that ChABC with trehalose in solution led to significant increases in enzymatic activity over a two week period compared to ChABC without trehalose in solution.

To study the release from lipid microtubes, either F-ChABC or unmodified ChABC was dissolved in 1 M trehalose at a concentration of 1 mg/mL or 500 mU/mL respectively, then loaded into lyophilized microtubes. The loaded lipid microtubes were encapsulated into 10 mg/mL fibrin scaffolds and allowed to release into a 1 M trehalose solution over 14 days. Release of F-ChABC was analyzed using a fluorimeter and the activity of ChABC was measured using an enzymatic activity assay to determine the amount of active units released from the fibrin scaffolds. The lipid microtubes allowed for sustained release of F-ChABC over 14 days compared to F-ChABC that was directly loaded into fibrin scaffolds, which released of over 85% of the F-ChABC in 7 days (Figure 2.9B).

The ability to release active ChABC was measured from lipid microtubes encapsulated into fibrin scaffolds. ChABC loaded directly into fibrin scaffolds without lipid microtubes showed a total release of over 40% of F-ChABC on day 1 (Figure 2.9B), but only 8.7±3.9% of the enzyme remained active after release on day 1 (Figure 2.9C) and limited enzymatic activity was measured thereafter. In contrast, loading thermostabilized ChABC into lipid microtubes prior to encapsulation in fibrin scaffolds led to an initial release of 18.8±2.2% of F-ChABC on day 1 (Figure 2.9B) and 23.6±4.3% of the total loaded active enzyme was released on day 1 (Figure 2.9C). Enzymatic activity was detected up to day 10 with limited activity between 10 and 14 days. Overall, 50.7±4.0% of active ChABC was released from fibrin scaffolds with microtubes by day 14. Thus thermostabilized bioactive ChABC can be loaded and released from lipid microtubes for over one week *in vitro*.

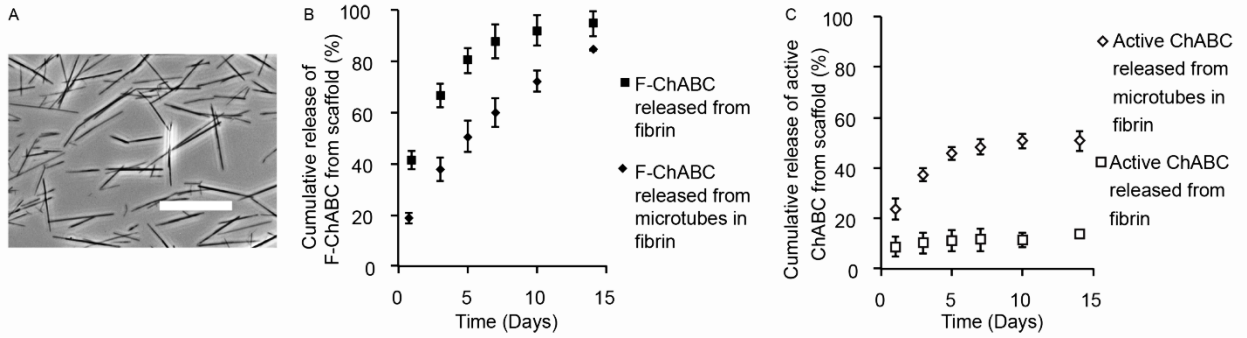

Figure 2.9: Incorporation of ChABC into lipid microtubes and fibrin scaffolds. (A) Phase contrast image of lipid microtubes. Average length measured 21±14 μm. Scale bar is 25 μm. **(B)** Fluorescent measurements of F-ChABC released from lipid microtubes within fibrin scaffolds or from F-ChABC in fibrin scaffolds alone (without microtubes). Loading efficiency of ChABC in microtubes was 89.5±4.7%. Loading F-ChABC into lipid microtubes allows for sustained release compared to adding F-ChABC directly into fibrin scaffolds. **(C)** Open data points are enzymatic activity measurements of ChABC and closed data points are fluorescence measurements of F-ChABC. Comparison between the enzymatic assay and fluorescence measurements suggests that the amount of released and active ChABC is similar to the release rate of F-ChABC loaded lipid microtubes for one week. In contrast, the amount of enzymatically active ChABC released when directly loaded into fibrin scaffolds shows a much lower release rate, suggesting that ChABC loses activity during the formation of the fibrin scaffolds when not protected within the lipid microtubes. ChABC activity was measured for up to 10 days after scaffold formation.

2.5 Discussion

After SCI, upregulation of extrinsic factors including CSPGs and MAIs affect functional recovery by limiting axonal outgrowth and preventing reformation of functional synapses (Sharma et al., 2012). Here we demonstrate that combination treatment with ChABC and NEP1-40 enhances neurite extension compared to either treatment alone when dissociated chick DRGs are cultured on inhibitory spot assays containing CSPGs and MAIs. Furthermore, active ChABC and NEP1-40 can be loaded and released from dual drug delivery systems incorporated into fibrin scaffolds.

CSPGs induce outgrowth inhibition through interactions of glycosaminoglycan chains with several receptor proteins found on neurons including PTPσ, LAR, NgR1, and NgR3 by initiating the Rho/ROCK signaling cascade (Shen et al., 2009, Fisher et al., 2011, Dickendesher et al., 2012). CSPGs may also block growth promoting mechanisms by interfering with integrin signaling to further limit axonal growth potential (Zhou et al., 2006). In addition, CSPGs are known to contribute to formation of perineuronal nets that limit axonal outgrowth and control plasticity in the central nervous system. Deposition of CSPGs after injury is significantly upregulated by reactive astrocytes that form the glial scar. The time course of CSPG deposition is varied based on the type of CSPG, with expression levels typically peaking between 1 and 2 weeks and continued expression up to 4 weeks or more after injury (Jones et al., 2003). Removal of inhibition via enzymatic cleavage of the glycosaminoglycan chains from the core protein with bacterially-expressed ChABC has consistently shown robust increases in neuronal outgrowth, although the core protein has some remaining inhibitory effect (Bradbury et al., 2002a). Our data further verifies that degradation of CSPGs using ChABC leads to increased neurite extension. Cells cultured on myelin spots alone did not have increased neurite extension with the addition of ChABC, while dual spots with myelin and CSPGs did have increased extension, but the outgrowth was not as robust as CSPGs alone. This

distinction suggests that ChABC is capable of removing inhibition due to CSPGs but does not remove inhibition from MAIs.

The myelin-associated membrane proteins, such as Nogo-66, MAG, and OMgp, bind the Nogo receptor on neurons, which triggers the Rho/ROCK cascade through Lingo-1 and p75 transmembrane proteins (Liu et al., 2002, Wang et al., 2002a, McGee and Strittmatter, 2003). Blocking the upstream activation of the ROCK signaling pathway can be achieved using Nogo receptor fragments, targeted Nogo receptor antibodies, or inactivating downstream effectors of the pathway (Liebscher et al., 2005a, Forgione and Fehlings, 2013). Similarly, limiting the activation of RhoA, RhoB, and RhoC proteins increased functional recovery in animal models (Dergham et al., 2002, Fournier et al., 2003, Fu et al., 2007). Myelin inhibitory spots significantly decreased neurite extension of dissociated chick DRGs, and treatment with NEP1-40 allowed the neurites to overcome the inhibition and extend processes.

NEP1-40 is a small peptide sequence consisting of 40 amino acids, thus it diffuses away from the local injury site and can be taken up and degraded by infiltrating macrophages and microglia. Therefore, it is necessary to provide sustained delivery of the peptide to the injured spinal cord to continually block myelin-based inhibition. Another popular method to limit activation of the Nogo receptors is the use of anti-Nogo-A antibodies, which have been shown to improve functional recovery following SCI. Most treatments using anti-Nogo-A antibodies or NEP1-40 began treatment immediately after injury and typically continued for 2 weeks but delivered for up to 4 weeks post-injury (GrandPre et al., 2002, Liebscher et al., 2005b, Maier et al., 2009, Zhao et al., 2013). For example, intrathecal pump delivery of anti-Nogo-A antibodies for 2 weeks used in combination with ChABC treatment showed improved recovery over either treatment alone (Zhao et al., 2013). Therefore, sustained delivery of NEP1-40 over 2-4 weeks may be a target timeframe for

SCI treatment. The 50:50 monomer ratio and low intrinsic viscosities were both chosen because of previous work showing these parameters allow for relatively rapid degradation of the microspheres and rapid release of the loaded molecule, although smaller diameter microspheres may further increase the degradation rates (Beck et al., 1980, Kamei et al., 1992, Okada and Toguchi, 1995, Anderson and Shive, 2012). Future research may benefit from adjusting the release profiles to explore the effect of the timing and duration of NEP1-40 treatment on regeneration in vivo.

Therapies combining ChABC and anti-myelin inhibitory molecules have shown varied results. Treatment of a thoracic contusion injury with ChABC, anti-Nogo-A antibodies, and treadmill training resulted in significant functional improvements over any single treatment, suggesting a synergistic effect with combination therapy (Zhao et al., 2013). However, another study reported combined treatment with ChABC and NEP1-40 did not increase axon growth in organotypic co-cultures taken from neonatal rats compared to either treatment alone, which suggests that the combined treatment may be limited because CSPGs and MAIs affect some of the same intracellular pathways (Nakamae et al., 2010). Furthermore, several studies have shown that using ChABC or other anti-inhibitory molecules in severe SCI models lead to increased functional recovery, but moderate or mild injury models have limited or no significant recovery compared to untreated controls (Caggiano et al., 2005, Mitsui et al., 2005a). Therefore, it may be necessary to use adult animals and severe injury models in order to demonstrate that combination treatment with ChABC and NEP1-40 improves recovery over either treatment alone. However, treatment with ChABC and NEP1-40 did not promote neurite extension to levels seen outside of the inhibitory spots. It may be beneficial to try various combinations of treatments that target both the intrinsic growth potential and extrinsic inhibitory environment to further improve extension and regeneration (Gopalakrishnan et al., 2008, Blesch et al., 2012, Walker et al., 2012, Ma et al., 2014).

Delivery of active anti-inhibitory molecules to the injured spinal cord is typically achieved using invasive intrathecal pumps or cannulas and microinjections, which can cause increased scarring and compression of the spinal cord (Jones and Tuszynski, 2001). The ability to deliver active drugs to the injury using an implanted biomaterial scaffold may provide an improved alternative drug delivery method. PLGA microspheres were chosen to release the NEP1-40 peptide for their well-established formation methods, which allow for tunable control over the release rate and a longer release period (Jain, 2000, Yang et al., 2000a, Kim et al., 2008a). The release rate is dependent upon the rate of hydrolytic degradation of the PLGA microspheres, which can be controlled by altering the ratio of lactic acid to glycolic acid, the intrinsic viscosity of the polymer (related to the molecular weight), or by changing formation conditions. PLGA with low intrinsic viscosity was chosen because decreasing the intrinsic viscosity increases the degradation and release rate of the microspheres. The PLGA microspheres formed had lower loading efficiencies but comparable release profiles to previously published research on delivery of alkaline phosphatase (Kim et al., 2008a). The ChABC enzyme was also tested with PLGA microspheres, but no enzymatic activity was measured after loading and release due to the highly labile and thermally unstable nature of the enzyme especially in organic solvents. The quantity of PLGA microspheres and lipid microtubes added to the scaffolds may be augmented to increase the amount of anti-inhibitory molecules delivered to the spinal cord. However, an upper limit will be reached when the amount of drug delivery systems begins to negatively affect the gelation of the fibrin scaffolds and potentially inhibit the ability of fibrin to form a stable hydrogel.

In order to maintain ChABC activity after release, the enzyme must be thermally stabilized and loaded into a delivery system under mild conditions. Stabilization of protein structure, using naturally occurring trehalose, is used to maintain activity (Yager and Schoen, 1984, Crowe et al., 1988, Rhodes et al., 1988). In the absence of trehalose, bioactivity of ChABC drops dramatically *in*

vitro. Stabilized enzymes can be loaded into lyophilized lipid microtubes, embedded into biomaterial scaffolds, and upon release the enzyme is inactivated under normal deactivation kinetics (Meilander and Yu, 2001, Johnson et al., 2009, Lee et al., 2009). For instance, the activity of TGF-β was shown to persist for up to 10 hours when released from microtubes, while the half-life of TGF-β *in vitro* is on the order of minutes (Spargo et al., 1995). Furthermore, the loading of ChABC led to maintained activity over 2 weeks while unstabilized ChABC lost activity after a few days (Lee et al., 2009). ChABC added directly into fibrin scaffolds without prior loading into microtubes resulted in a loss of over 85% of ChABC activity in the first day, likely due to the rapid diffusion of trehalose away from ChABC causing normal deactivation kinetics. Interestingly, ChABC loaded directly into highly concentrated fibrin scaffolds was shown to maintain activity in vitro and in vivo, although, the fibrin used was 10 times more concentrated than the fibrin used was 10 times more concentrated than the fibrin reported here (Hyatt et al., 2010).

2.6 Conclusion

In conclusion, research has shown the promising effects of treating SCI with ChABC and anti-myelin associated inhibitors such as NEP1-40, but SCI is complex with a multitude of inhibitory cues that must be overcome to improve functional recovery. Therefore, it may be necessary to develop a treatment that is capable of providing mechanisms that eliminate or mitigate multiple obstacles. We have developed a dual drug delivery system capable of providing sustained release of ChABC from lipid microtubes and NEP1-40 from PLGA microspheres. The drug delivery system is capable of being embedded into fibrin scaffolds, which our lab has previously used to deliver growth factors and neural progenitor cells to the injury site, and removes the need for invasive intrathecal pumps or catheters. The following chapter will use the materials developed in combination with the delivery of specific neural progenitors and growth factors to synergistically aid in recovery after SCI.

Chapter 3

Anti-Inhibitory Drug Delivery Systems Co-transplanted with Progenitor Motor Neurons in a Rat Spinal Cord Injury Model

3.1 Abstract

Regeneration of lost synaptic connections following spinal cord injury (SCI) is limited by local ischemia, cell death, and an excitotoxic environment, which leads to the development of an inhibitory glial scar surrounding a cystic cavity. While a variety of single therapy interventions provide incremental improvements to functional recovery after SCI, they are limited; a multifactorial approach that combines several single therapies may provide a better chance of overcoming the multitude of obstacles to recovery. To this end, fibrin scaffolds were modified to provide sustained delivery of neurotrophic factors and anti-inhibitory molecules, as well as encapsulation of embryonic stem cell-derived progenitor motor neurons (pMNs). The efficacy of the scaffolds, prior to transplantation, was established by validating pMN viability and differentiation was unaffected by culture within scaffolds with sustained delivery of anti-inhibitory molecules. *In vitro* characterization of this combination scaffold confirmed that pMN viability was unaffected by culture alongside sustained delivery systems. When transplanted into a rat sub-acute SCI model, fibrin scaffolds containing anti-inhibitory molecules without pMNs were capable of removing proteoglycans within

the glial scar. Scaffolds containing pMNs, but not anti-inhibitory molecules, showed survival, differentiation into neuronal cell types, axonal extension in the transplant area, and the ability to integrate into host tissue. However, the combination of pMNs with sustained-delivery of anti-inhibitory molecules led to reduced cell survival and increased inflammation. While combination therapies retain potential for effective treatment of SCI, further work is needed to improve cell survival and to limit inflammation.

3.2 Introduction

Spinal cord injury (SCI) typically results from mechanical trauma that severs axons, damages vasculature, and promotes a secondary injury that results in cell death, leading to the formation of a cystic cavity and inhibitory glial scar, which act as chemical and physical barriers to regeneration. The variety of local and systemic changes caused by SCI creates a multitude of obstacles limiting recovery, which together hinder the effectiveness of single therapeutic interventions. This has prompted the development of combination strategies that synergistically work together to improve recovery.

One major therapeutic strategy following SCI is cell transplantation. A wide variety of cell types have been investigated with emphasis placed on transplanting cells that are relevant to the central nervous system (CNS), such as astrocytes, oligodendrocytes, and neurons (Davies et al., 2011, Tetzlaff et al., 2011, Ruff et al., 2012, Wu et al., 2012, Chu et al., 2014). Embryonic stem cells (ESCs) induced to form neural progenitor cells are promising, but have poor survival post-transplantation (~10% in the absence of scaffold); cells that do survive typically differentiate into glia with poor neuronal differentiation and maturation (Cao et al., 2002, Mitsui et al., 2005b, Karimi-

Abdolrezaee et al., 2006, Neuhuber et al., 2008, Parr et al., 2008). Furthermore, one significant drawback to the use of ESC-derived neural cells is the potential formation of teratomas due to the presence of undifferentiated ESCs in the transplant (Johnson et al., 2010b). To address these concerns, differentiation protocols have been developed to obtain more restricted progenitor populations including progenitor motor neurons (pMN), which give rise to motoneurons, oligodendrocytes and type II astrocytes (Erceg et al., 2009, Rossi et al., 2010). To further improve the purity of ESC-derived cells, our lab has developed a transgenic ESC line (P-Olig2) that expresses antibiotic resistance under the lineage-specific pMN marker, Olig2, which allows for positive selection of ESC-derived pMNs after differentiation (McCreedy et al., 2014).

Another therapeutic strategy is the delivery of pro-regenerative neurotrophic factors that are normally secreted by local glia and are vital to the maintenance of healthy tissue. Following injury, specific factors are upregulated within the spinal cord and help reduce cell death. Exogenous delivery of a variety of neurotrophic factors has been shown to increase cell survival and limit formation of the glial scar (Schnell et al., 1994a, Grill et al., 1997a, Grill et al., 1997b, Ye and Houle, 1997, Jakeman et al., 1998, Menei et al., 1998, Liu et al., 1999, Storer et al., 2003, Taylor et al., 2006a). Delivery of platelet-derived growth factor (PDGF)-AA can aid stabilization and maturation of blood vessels at the injury site and is an important factor in oligodendrocyte maturation (Raff et al., 1983, Hart et al., 1989, Almad et al., 2011), while neurotrophin 3 (NT-3) promotes regeneration of the corticospinal tract and dorsal sensory axons (Schnell et al., 1994a, Grill et al., 1997a). When NT-3 and PDGF-AA were delivered from modified fibrin scaffolds capable of sustained release alongside ESC-derived pMNs, there was an increase in expression of neuronal markers both rostral and caudal to the injury site and improved migration of transplanted cells into the host spinal cord (McCreedy et al., 2014).

A third therapeutic approach is to limit the effects of the inhibitory environment surrounding the injury site using anti-inhibitory molecules. Two major inhibitory factors that are significantly upregulated after SCI are chondroitin sulfate proteoglycans (CSPGs) and myelin-associated inhibitors (MAIs), which form a biochemical barrier to axon growth (McGee and Strittmatter, 2003, Kwok et al., 2012). Under normal conditions, CSPGs are major components of perineuronal nets and are found within CNS extracellular matrix (Kwok et al., 2011). Inhibition by CSPGs is caused by the glycosaminoglycan (GAG) side chains, which are able to interact with target receptors on neuronal membranes (Shen et al., 2009, Fisher et al., 2011, Dickendesher et al., 2012). Treatment with chondroitinase ABC (ChABC) cleaves the GAG side chains and prevents activation of target receptors (Zuo et al., 1998, Moon et al., 2001, Bradbury et al., 2002b, Garcia-Alias et al., 2008, Garcia-Alias et al., 2009, Wang et al., 2011).

MAIs, such as Nogo-A, oligodendrocyte-myelin glycoprotein, and myelin-associated glycoprotien, are important chemical cues in the mature CNS that limit axon growth and stabilize functional neuronal circuits (Wang et al., 2002b, Schnaar, 2010). However, following SCI MAIs are not degraded or removed from the injury site, which results in limited axonal sprouting and regeneration (Lee and Zheng, 2012). Most MAIs interact with neurons through a common receptor, the Nogo receptors (NgR1 and NgR3), which initiate the Rho/ROCK cascade resulting in microtubule disassembly and growth cone collapse (Fournier et al., 2001, Domeniconi et al., 2002a, Liu et al., 2002, Wang et al., 2002a). Although MAIs bind other receptors, inhibition of NgR1 using a small competitive antagonist (NEP1-40) significantly improved neurite outgrowth on myelin inhibitory substrates and improved functional recovery in rat SCI models (GrandPre et al., 2002). Both types anti-inhibitory molecules are typically delivered using invasive intrathecal pumps, catheters, or microinjections, which have the potential for increased scarring and compression of the spinal cord (Jones and Tuszynski, 2001). Drug delivery using anti-inhibitory microparticle systems

(AIMS) within fibrin scaffolds are able to provide sustained local delivery of ChABC and/or NEP1-40 in a minimally invasive way. Using AIMS to deliver ChABC and NEP1-40, decreased CSPG deposition and increased axon growth were observed in a rat acute SCI model (Wilems – JCR Submitted).

The combined delivery of cells, neurotrophic factors, and AIMS may lead to improved recovery compared to any single treatment alone. To this end, we incorporated ESC-derived pMNs and sustained drug delivery systems for anti-inhibitory molecules and neurotrophic factors into fibrin scaffolds and tested their efficacy in both in vitro and in vivo models. To confirm their potential prior to transplantation, pMNs were cultured within biomaterial scaffolds containing the drug delivery systems for two weeks to measure cell viability in vitro. The combination scaffolds were then transplanted into a sub-acute rat SCI model to repopulate cells lost following injury, increase host and transplant cell survival, and reduce the local inhibitory environment. Transplantation of AIMS within fibrin scaffolds did not affect the presence of CSPGs within the glial scar compared to transplantation of pMNs with growth factors or growth factor delivery alone, but the presence of CSPGs increased when pMNs were combined with AIMS. While pMNs transplanted into fibrin scaffolds alone were viable and able to differentiate into neurons and astrocytes, a decrease in cell survival was observed when pMNs were transplanted in combination with AIMS, possibly due to elevated macrophage infiltration.

3.3 Materials and Methods

3.3.1 Acute Spinal Cord Injury and Immediate 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 (250-300 g) were anesthetized using 2-5% 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. The dura mater was removed from the exposed cord at T8. Vitrectomy scissors mounted to a micromanipulator were lowered 1.5 mm into the spinal cord. A lateral incision was created across the spinal cord to from a dorsal hemisection.

Following the initial incision, fibrin scaffolds with total volume of 200 μL were prepared by mixing 10 mg/ml fibrinogen, 2.5 mM CaCl2 (Sigma), 2 NIH units/ml thrombin (Sigma), 30 mg of NEP1-40-loaded PLGA microspheres (1.5 mg per transplant) and 10 mg of ChABC-loaded microtubes (0.5 mg per transplant). For fibrin control group, empty drug PLGA microspheres and lipid microtubes were incorporated into the fibrin scaffold as controls. 4 animals were used per group and 12 rats total survived the injury and transplantation out of 15. A 10 μL fibrin scaffold was allowed to polymerize for 5 minutes prior to implantation into the injury site. Using fine tooth forceps, the fibrin scaffold was gently forced into the dorsal hemisection incision. Following implantation, a second 10 μL fibrin scaffold was allowed to polymerize in situ to hold the delivery system/scaffold within the injury site, covered with artificial dura, then the overlying back muscles closed using degradable sutures, and the skin stapled closed.

Immediately following surgery, animals were given cefazolin (25 mg/kg) and buprenorphine (0.04 mg/kg). Cefazolin was continued twice daily for 5 days with buprenorphine (0.04 mg/kg) given twice daily for 3 days and (0.01 mg/kg) for the next 3 days. Bladders were manually expressed twice a day until normal bladder function was resumed. Two weeks following treatment, 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. Spinal cords were then cryoprotected in 30% sucrose in PBS. Prior to embedding, 3 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 (Leica CM1950).

3.3.2 Embryonic Stem Cell Culture

A previously established transgenic ESC line (P-Olig2) was used for transplantation (McCreedy et al., 2012). The P-Olig2 cell line contains the puromycin N-acetyltransferase (PAC) gene under the control of the native Olig2 regulatory elements in one allele, thus conferring puromycin resistance to cells expressing Olig2 after induction and allowing for positive selection of pMNs. To allow tracking of these cells, enhanced green fluorescent protein (GFP) was randomly inserted under the control of the beta-actin promoter to provide ubiquitous reporter expression throughout the experiments (Seiler-Tuyns et al., 1984, Quitschke et al., 1989, McCreedy et al., 2012, McCreedy et al., 2014). P-Olig2 ESCs were grown in complete media consisting of Dulbecco's modified Eagle's Medium (Life Technologies, Carlsbad, CA) supplemented with 10% newborn calf serum (Life Technologies), 10% fetal bovine serum (Life Technologies), and 1:200 100x EmbryoMax® Nucleosides (EMD Millipore, Billerica, MA). Cells were passaged at a 1:5 ratio every 2 days and seeded on a new T-25 flask coated with a 0.1% gelatin solution (Sigma). To avoid the use of a feeder cell layer, 1000 U/mL leukemia inhibitory factor (LIF; EMD Millipore) and 100 μM β-

mercaptoethanol (BME; Life Technologies) were added to the media to maintain ESCs in an undifferentiated state (Willerth et al., 2007, McCreedy et al., 2012).

3.3.3 Progenitor Motor Neuron Differentiation

For pMN induction, P-Olig2 ES cells were exposed to retinoic acid (RA; Sigma) and smoothened agonist (SAG; EMD Millipore) in a $2^-/4^+$ induction 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 (Life Technologies) supplemented with 5% knockout serum replacement (Life Technologies), 50 μM non-essential amino acids Life Technologies),1:100 100x Insulin-Transferrin-Selenium (Life Technologies), 1:200 100x EmbryoMax® Nucleosides. EBs were allowed to form for 2 days in the absence of induction factors (2^-). EBs were then cultured in DFK5 supplemented with 2 μM RA and 0.5 μM SAG for the final four days (4^+). Media was changed every 2 days. In selected cultures, 4 μg/mL puromycin (Sigma) was added during the final 2 days of induction (McCreedy et al., 2012)

3.3.4 Formation of Poly(lactic-*co*-glycolic acid) (PLGA) Microspheres:

PLGA microspheres, with a 50:50 lactic acid to glycolic acid ratio, were fabricated using a water in oil in water double emulsion solvent evaporation technique (Kim et al., 2008b). PLGA (10% w/v, intrinsic vis. = 0.15-0.3 dL/g, Absorbable Polymers Inc., Pelham, NJ) was dissolved in 2 mL dichloromethane (DCM). 100 μL of 10 mg/mL NEP1-40 (Mw = 4627 Da, Sigma) was added to the PLGA/DCM solution and sonicated for 10 seconds (Microson, Misonix Inc.), added to 25 mL of water with 1% w/v poly(vinyl alcohol) and 10% w/v NaCl and homogenized. The resulting emulsion was poured into 250 mL of water with 0.1% w/v poly(vinyl alcohol) and 10% w/v NaCl, then magnetically stirred for 3 hours, washed with water, frozen overnight at -80°C and lyophilized. PLGA microspheres were imaged using a scanning electron microscope (Nova NanoSEM 230, FEI)

after gold sputter coating for 45 seconds. Diameters from individual microspheres were measured using ImageJ software and 100 microsphere diameters were averaged per batch.

3.3.5 Formation of Lipid Microtubes:

Lipid microtubes were formed similar to previous work (Meilander et al., 2001). 1,2-bis(tricosa-10,12-diynoyl)-*sn*-3-phosphocholine ($DC_{8,9}PC$) lipids (Avanti Polar Lipids, Alabaster, AL) were dissolved at 55°C in 70% ethanol at 1 mg/mL and placed into a temperature controlled water bath (Haake A10, ThermoScientific, Asheville, NC). The total volume per batch was 5 mL. The temperature was decreased from 55°C to 25°C at a rate of 2.5°C/min. The lipid tubes were then stored at room temperature in the dark for one week. Trehalose (EMD Millipore) was added to a final concentration of 50 mM, the solution was centrifuged (1200 rcf, 5 minutes) to pellet the lipid microtubes, and 4 mL of solution was removed. The concentrated (5 mg/mL) microtube solution was lyophilized overnight to dry and stored at -20°C until further use. Prior to use 10 mg of dried microtubes were rehydrated with 100 μLs of 500 mU/mL ChABC dissolved in 1 M trehalose for 2 hours at 4°C. After loading with ChABC, the solution was diluted with 15 mLs of tris buffered saline, pH 7.4, then centrifuged (1200 rcf, 5 minutes) and the supernatant was removed. This step was performed one more time and the resulting loaded microtube pellet was used in 3D fibrin scaffold cultures or scaffold implantation into rat spinal cords.

3.3.6 3D Fibrin Scaffold Cultures

Fibrin scaffolds were prepared by dissolving fibrinogen (50 mg/mL; EMD Millipore) in TBS and dialyzing in 4L TBS for 24 hours. Fibrinogen was then sterile filtered before adjusting the concentration to 20 mg/mL as measured by UV spectroscopy. As previously described, a solution (150 μL) containing 10 mg/mL fibrinogen, 2.5 mM $CaCl_2$ (Sigma), and 2 NIH units/mL thrombin (Sigma) was mixed in a 48 well plate (Willerth et al., 2006, Willerth et al., 2007). For incorporation of

AIMS, the fibrin scaffolds were mixed with 45 mg NEP1-40-loaded PLGA microspheres and 7.5 mg ChABC-loaded lipid microtubes. All scaffolds were washed 5 times with 500 μL TBS per wash over a 24 hour period to remove unbound delivery system components (heparin and/or growth factors).

Three to five EBs containing pMNs were placed on each fibrin scaffold, and an additional fibrin scaffold (100 μL) with only $CaCl_2$ and thrombin was added to the top of the EBs. The 48 well plates containing fibrin scaffolds with EBs were incubated for 1 hour at 37°C. Cell media (500 μL) containing a 1:1 ratio of DFK5 media and Neurobasal (NB) (Life Technologies) media with 2% B27 supplement (Life Technologies) and 5 μg/mL aprotinin (Sigma) was then added to the top of the scaffolds. The EBs were cultured for 2 weeks with a single media change on day 3 to change to NB media with 2% B27 supplement and 5 μg/mL aprotinin.

3.3.7 Flow Cytometry

For evaluation of cell survival following 2 weeks of 3D culture, 0.25% trypsin-EDTA was added for 15 min to dissociate the EBs. pMNs from 2 scaffolds within the same group were combined together, triturated into a single cell suspension, then quenched with complete media. Cells were pelleted by centrifugation (5 min at 230xg), the supernatant was removed, and a standard LIVE/DEAD® Cell Viability Assay (Life Technologies) was performed. Live cells were identified by the incorporation of the membrane permeable calcein AM stain within a cell, whereas dead cells were identified by the binding of ethidium homodomer-1 to the nucleic acids of cells with damaged plasma membrane. Stained cells were analyzed for percent alive and dead using a Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For each group, 30,000 events were recorded and FloJo software (FloJo, Ashland, OR) was used for population gating following debris removal based on forward scatter versus side scatter. Results from flow cytometry are reported as the

percentage of cells staining positive for the live cellular marker or dead cellular marker out of the total cell population.

3.3.8 Sub-Acute Spinal Cord Injury and Delayed Scaffold Implantation

Experiments performed 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. Prior to surgery, animals (female Long-Evans rats, 250-275 g) were anesthetized with 5% isoflurane gas and an injection of 5 mg/kg xylazine. An incision was made through the back to expose the back muscle. Parallel cuts were made through the muscle on each side of the vertebral processes from T6-T10. To expose the spinal cord, the dorsal lamina was removed at T8 with fine tip rongeurs. The spinal cord was stabilized using spinal clamps placed in the vertebral foramina at T7 and T9. Dura mater was removed and vitrectomy scissors attached to a micromanipulator were lowered 1.5 mm into the spinal cord. Using the vitrectomy scissors, a lateral dorsal hemisection was performed on the spinal cord. Following removal of the vitrectomy scissors and micromanipulator, the spinal cord was covered with artificial dura, the back muscles closed with degradable sutures, and the skin was stapled closed. Animals were given cefazolin (25 mg/kg) and buprenorphine (0.04 mg/kg) (Quitschke et al., 1989). Cefazolin was continued twice daily for 5 days with buprenorphine (0.04 mg/kg) provided twice daily for 3 days and (0.01 mg/kg) for the next 3 days. Throughout the study, bladders were manually expressed twice a day until normal bladder function returned. Two weeks after the dorsal hemisection, the spinal cord was re-exposed, and a cavity for implantation was made by removing the scar tissue. Multifactorial fibrin scaffolds (300 μL) were prepared by mixing 10 mg/mL fibrinogen, 2.5 mM $CaCl_2$, 2 NIH units/mL thrombin, 62.5 μM heparin, 0.25 mM ATIII peptide, 125 ng NT3, 20 ng PDGF-AA, 45 mg NEP1-40-loaded microspheres, and 15 mg ChABC-loaded microtubes per scaffold., The ATIII bi-domain peptide

(GNQEQVSPKβAFAKLAARLYRKA), synthesized by solid phase Fmoc chemistry as previously described, was used to provide sustained growth factor delivery (GFs) (Sakiyama-Elbert and Hubbell, 2000a, b). For scaffolds containing growth factors heparin, ATIII peptide, and growth factors were added to each fibrin scaffold. For scaffolds containing GFs and pMNs, the microspheres and microtubes were excluded from the formation solution. For scaffolds containing AIMS and pMNs, heparin, ATIII peptide, and growth factors were excluded from the formation solution. For scaffolds not containing cells, the pMNs were excluded from the formation solution (Table 3.1).

Table 3.1: Design of SCI combination therapy study. All groups had fibrin as the scaffold and included some combination of heparin, ATIII peptide, GFs (NT-3 & PDGF), NEP1-40 PLGA microspheres, ChABC lipid microtubes, and pMNs.

Combination Scaffolds	Heparin	ATIII peptide	NT-3/PDGF	NEP1-40 PLGA microspheres	ChABC lipid microtubes	Selected pMNs
GFs (n=5)	X	X	X			
pMNs+GFs (n=5)	X	X	X			X
AIMS (n=6)				X	X	
pMNs+AIMS (n=7)				X	X	X
GFs+AIMS+pMNs (n=6)	X	X	X	X	X	X

For implantation, a 10 μL fibrin scaffold was polymerized for 5 min then implanted into the created cavity. For groups containing pMNs, 10 EBs were added to the 10 μL scaffold during polymerization. A second 10 μL fibrin scaffold containing only CaCl2 and thrombin was polymerized in situ to secure the initial scaffold within the lesion cavity. Following implantation, the spinal cord was covered with artificial dura, the back muscle closed with sutures, and the skin stapled closed. Upon completion of the surgery, animals were given buprenorphine and cefazolin in the same doses as specified for the initial injury. Bladders were manually expressed twice a day until normal bladder function returned. To limit immune rejection of the mouse cells, daily injections of

cyclosporine-A (10 mg/kg, Novartis) were given. Two weeks after scaffold implantation, animals were euthanized by an overdose of Euthasol, and a transcardial perfusion performed with 4% paraformaldehyde. Spinal cords were harvested and post-fixed in 4% paraformaldehyde for 24 hours. After fixing, cords were cryoprotected in 30% sucrose in 10 mM PBS. Cords were embedded in Tissue-Tek OCT compound, frozen, and cut into 20 μm sagittal sections with a cryostat (Leica CM1950).

3.3.9 Immunohistochemistry:

To determine expression of markers at the site of injury, immunohistochemistry was performed on 6 spinal cord sections per animal. OCT was washed from spinal cord 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 PBS with 2% NGS: β-tubulin III (β-tubIII, Covance, Dedham, MA, 1:400), glial fibrillary acidic protein (GFAP, ImmunoStar, 1:100), chondroitin sulfate (CS56, Sigma, 1:250), CD68 (ED1, AbD Serotec, 1:200), neuronal nuclei (NeuN, EMD Millipore, 1:500). Primary antibody staining was followed by 3 washes with PBS. Appropriate Alexa Fluor secondary antibodies (Life Technologies) in PBS with 2% NGS were applied for 2 hours at room temperature followed by an additional 3 washes in PBS. Sections were mounted using ProLong Gold anti-fade reagent with DAPI (Life Technologies).

3.3.10 Image Analysis of Immunohistochemistry:

To quantify the staining of markers at the injury site, a series of images spanning the lesion site were captured using a MICROfire camera attached to an Olympus IX70 inverted microscope using a 4x objective. As previously described, the images were merged using Adobe Photoshop, and the lesion site traced and expanded 250 μm away from the injury (McCreedy et al., 2014). The

average pixel intensity within the lesion area or 250 μm from the lesion border was measured using a custom Matlab (Mathworks, Natick, MA) script that determined the intensity of each individual pixel and then averaged all the intensities of pixels within the defined area.

3.3.11 Statistical Analysis:

In vitro data was analyzed by ANOVA followed by Scheffe's post-hoc test with a significance criterion of 95%. Statistical significance for *in vivo* studies was determined by using the non-parametric Kruskal-Wallis one-way analysis of variance with a significance criterion of 95%.

3.4 Results

3.4.1 Loaded PLGA Microspheres and Lipid Microtubes Limit Formation of the Glial Scar and Promote Axonal Extension in an Acute Dorsal Hemisection Injury:

PLGA microspheres loaded with NEP1-40 and lipid microtubes loaded with thermostabilized ChABC were incorporated into fibrin scaffolds and implanted into a thoracic dorsal spinal cord hemisection immediately after injury. Two weeks after treatment, the spinal cords were harvested, sectioned, and stained for several markers to analyze the effect of the drug delivery systems at the site of injury (Figure 3.1A). The injury group had the thoracic hemisection with no treatment following the injury (n=4). The fibrin group underwent the thoracic hemisection and was acutely treated with a fibrin scaffold containing empty PLGA microspheres and lipid microtubes (n=4). The treatment group had a thoracic hemisection that was acutely treated with a fibrin scaffold containing NEP1-40-loaded PLGA microspheres and ChABC-loaded lipid microtubes (n=4). PLGA microspheres remained within the transplant site and appeared as spherical voids within the

spinal cord sections, no evidence of lipid microtubes were seen within the sections. The loaded drug delivery systems promoted a significant decrease in the staining against CS56 (intact CSPGs, as assessed by average pixel intensity values) within 500 μm of the injury when compared to both injury alone and fibrin with empty microspheres and microtubes (Figure 3.1B). To measure neural fibers, antibodies against β-tubIII, a microtubule found in neurons, showed a significant increase in the average pixel intensity within 500 μm compared to the fibrin with empty delivery systems group although no difference was measured between the fibrin alone group and the fibrin with loaded microspheres and microtubes group.

Furthermore, the development of the glial scar was quantified by staining for GFAP, an intermediate filament found within astrocytes and upregulated in reactive astrocytes. Staining for GFAP showed that the fibrin with loaded delivery systems had significantly decreased GFAP staining compared to the fibrin with empty delivery systems group. The proof of concept animal study suggests that the sustained dual delivery of ChABC and NEP1-40 in an acute dorsal hemisection model may limit the presence of CSPGs, increase neuronal fiber extension, and affect the development of the glial scar after injury.

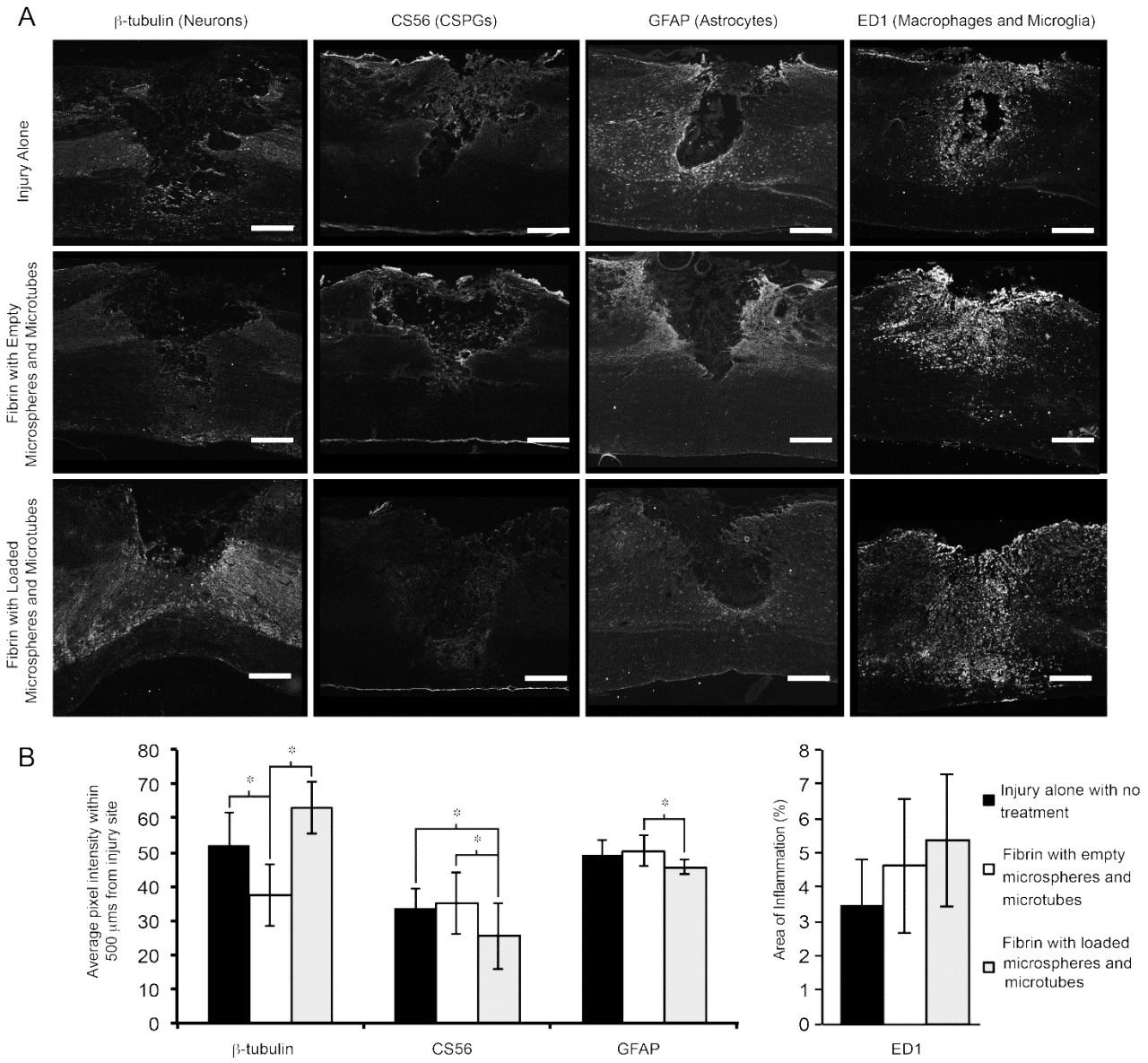

Figure 3.1: Scaffold transplantation following a thoracic dorsal hemisection model of spinal cord injury. (A) Immunohistochemistry of injured spinal cord sections two weeks after transplantation, 20 μm thick sections were stained with antibodies against CS56 (marker for CSPGs), ED1 (macrophages and microglia), GFAP (astrocytes and glial scar), or β-tubIII (neurons). **(B)** The average pixel intensity within 500 μm of the injury site was determined. Fibrin scaffolds with loaded delivery systems had increased β-tubIII intensities compared to fibrin with unloaded delivery systems. Deposition of CSPGs was decreased in the treated groups compared to the injury alone and fibrin with unloaded delivery systems. GFAP intensity was decreased in the fibrin with loaded delivery system groups. The percent area of inflammation was measured as the fraction of pixels that positively stained for ED1 in a 2 cm section. No significant changes were seen in the immune response between any groups. Error bars are standard deviation. * denotes significant difference between groups ($p < 0.05$). Scale bar is 250 μm.

3.4.2 pMN Survival is not Effected by Combination Therapy with AIMS *in vitro*:

To study the effect of AIMS on the viability of pMNs in long-term culture, puromycin-selected pMNs were cultured in 3D fibrin scaffolds containing either one or both AIMS for two weeks post-induction. A cell viability assay with flow cytometery was used to quantify cell survival (Figure 3.2). Fibrin scaffolds alone contained 93.4% live cells and 6.6% dead cells, while the inclusion of AIMS resulted in 90.1% live cells and 9.9% dead cells. No statistical differences were measured between the groups. Separating out the two AIMS resulted in 85.1% live cells and 14.9% dead cells when only ChABC-loaded lipid microtubes were included in fibrin scaffolds, and 89.1% live cells and 10.9% dead cells when only NEP1-40-loaded PLGA microspheres were included in fibrin scaffolds. Therefore, the AIMS do not directly affect survival of pMNs after two weeks in culture *in vitro*.

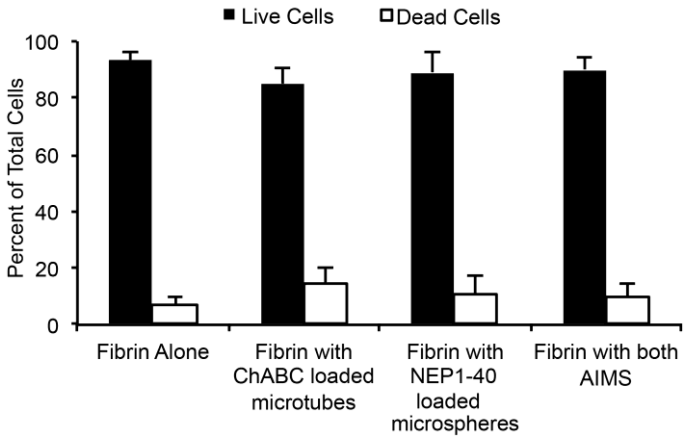

Figure 3.2: Viability of purified pMNs cultured in fibrin scaffolds with or without AIMS at two weeks *in vitro*. Flow cytometery quantification of the percent of live and dead cells after degradation of scaffolds. Live cells stained positive with calcein AM and dead cells stained positive for ethidium homodimer-1. No significant differences were seen between groups. Error bars are standard deviation.

3.4.3 Fibrin Scaffolds with AIMS decrease pMN Survival in a Sub-Acute Rat Injury Model:

Following *in vitro* analysis, fibrin scaffolds containing pMNs, the AIMS and the GF delivery system were transplanted into a sub-acute rat thoracic SCI model. Transplantation of the biomaterial scaffolds was delayed until two weeks after injury to allow stabilization of the lesion site and to improve transplanted cell survival. Five different groups were used to evaluate the effects of using combination therapies for SCI: (1) fibrin scaffolds with the heparin-based delivery of growth factors (GFs, n = 5), (2) fibrin scaffolds incorporating GFs with pMNs (GFs+pMNs, n=5), (3) fibrin scaffolds incorporating AIMS (AIMS, n=6), (4) fibrin scaffolds incorporating AIMS with pMNs (AIMS+pMNs, n=7), and (5) fibrin scaffolds incorporating GFs, AIMS, and pMNs (GFs+AIMS+pMNs, n=6). The transplanted pMNs ubiquitously express GFP under the β-actin promoter to allow visualization of transplanted cells.

Fluorescence imaging two weeks post-transplantation confirmed survival of GFP^+ cells in all groups that contained pMNs within the fibrin scaffolds (Figure 3.3A-C). However, the total GFP^+ area within the spinal cords was significantly different between groups with and without AIMS (Figure 3.3D). The GFP^+ area was decreased 10-fold when AIMS were included, suggesting AIMS have a negative effect on cell viability. While examining infiltration of inflammatory cells into and around the lesion site, large numbers of $ED1^+$ macrophages/microglia was observed when both pMNs and AIMS were included in the scaffolds. The lowest level of $ED1^+$ staining was seen in the AIMS alone and the GFs alone groups (Figure 3.4). Together these data suggest that the combination of AIMS and pMNs may lead to decreased transplanted cell survival due to an increased inflammatory response.

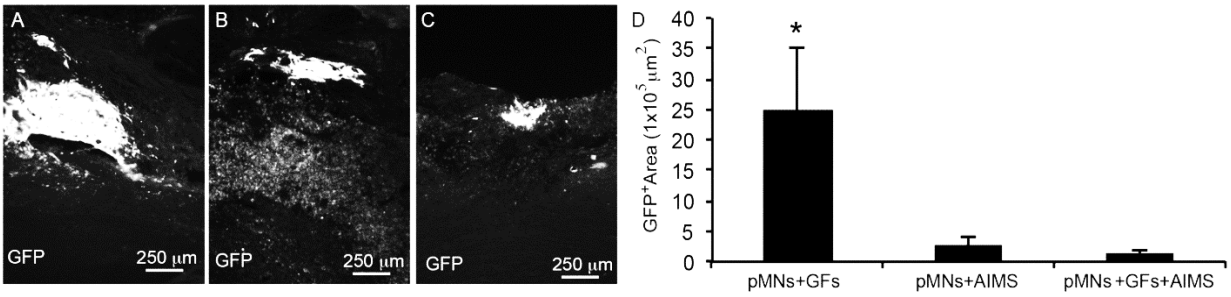

Figure 3.3: Effect of combination therapies on transplanted pMNs in injured spinal cord sections two weeks after transplantation. (A-C) Representative images of GFP+ areas within the (A) pMNs+GFs group, (B) pMNs+AIMS group, and (C) pMNs+GFs+AIMS group. **(D)** The average GFP+ area within the lesion site was determined. GFP expression was significantly decreased in groups containing AIMS compared to the pMNs+GFs group. Error bars are standard deviation. * denotes significant difference between other groups ($p<0.05$).

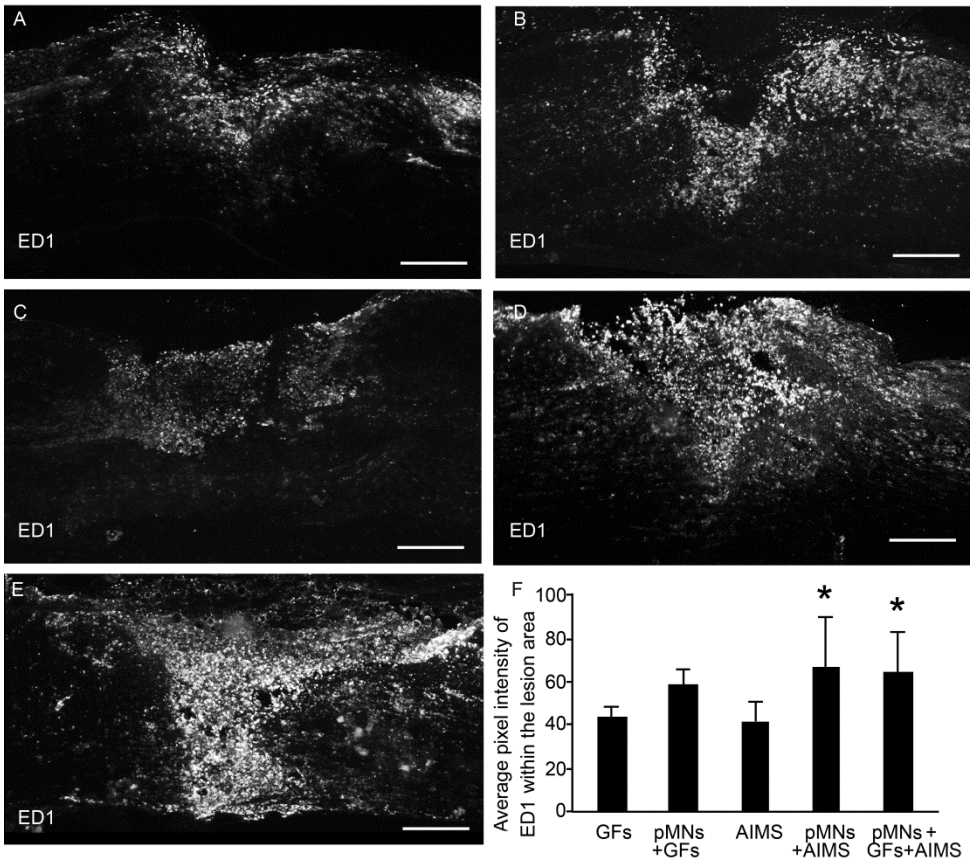

Figure 3.4: Effect of combining pMNs and AIMS on macrophage infiltration as assessed by ED1 staining. (A-E) Representative images of 20 μm thick sections of ED1 (marker for activated inflammatory cells) staining within and around the lesion site for groups containing (A) GFs, (B) pMNs+GFs, (C) AIMS, (D) pMNs+AIMS, (E) pMNs+GFs+AIMS. Positive staining for ED1 appears higher in groups containing both pMNs and AIMS. (F) The average pixel intensity of ED1 stained sections within the lesion area was determined. Fibrin scaffolds with pMNs+AIMS had significantly higher ED1 staining compared to AIMS. Error bars are standard deviation. * denotes significant difference versus the AIMS group ($p<0.05$). Scale bar is 250 μm.

3.4.3 Neural Differentiation and Integration of Transplanted pMNs following SCI:

Differentiation and axon extension of the surviving transplanted pMNs into mature neural cell fates was examined to determine if the transplanted cells were capable of becoming the appropriate cell types. Astrocytes and neurons were seen within GFP^+ areas. Astrocytes were visualized by staining for GFAP, with the strongest GFAP expression found at the border of the lesion and at the edges of the cell transplants (data not shown). No significant differences in GFAP expression was found between any groups, suggesting similar degrees of reactive gliosis. GFP^+ areas also stained strongly for neuronal nuclei (Figure 3.5). The pMNs+GFs group had significantly higher amounts of NeuN staining than other groups likely due to higher pMN survival. However, normalizing the number of NeuN+ cells in GFP^+ areas by the GFP^+ area no difference in the density neuronal nuclei (rough measure of the % of neurons from pMNs) between groups with transplanted cells was observed (Figure 3.6F). No NeuN+ cells were seen within the injury area in groups not receiving transplanted pMNs. Staining for the oligodendrocyte marker, O4, was inconclusive (data not shown).

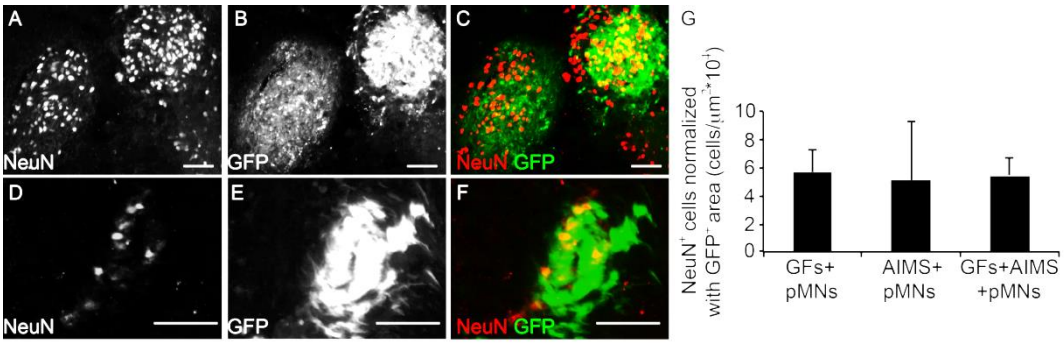

Figure 3.5: pMNs differentiate into neurons when transplanted into the injured spinal cord. (A-C) Representative images from GFs+pMNs and GFs+AIMS+pMNs groups stained with the neuronal nuclei marker, NeuN, two weeks post-transplantation (A-C) GFs+pMNs group showing $NeuN^+$ cells (A) and GFP^+ areas (B) that colocalize together (C). (D-F) GFs+AIMS+pMNs groups also stained positive with NeuN (D) within GFP^+ areas (E) and showed colocalization (F). The GFs+pMNs group had significantly higher GFP^+ areas and higher amounts of $NeuN^+$ cells but when normalized to the GFP^+ area showed similar levels of NeuN expression between all groups. Scale bars are 100 μm. Error bars are standard deviation.

In groups that contained transplanted pMNs, staining for the neuronal cytoskeletal protein β-tubIII demonstrated robust axonal extension within the transplant area (Figure 3.6A). The pMNs+GFs group had the greatest level of axonal extension within the lesion area, significantly higher than all other groups (Figure 3.6B). The groups containing pMNs+AIMS (with or without GF) showed a high degree of colocalization between β-tubIII and GFP expression, suggesting that the transplanted cells were capable of differentiating into neurons and extending axons. However, no differences in the total amount of β-tubIII were seen when compared to groups without pMNs, suggesting that a lower number of cells were surviving in the presence of AIMS compared to the pMNs+GFs group. Axon density in the host tissue immediately surrounding the lesion was not significantly different between any groups. In many cases where transplanted cells were adjacent to the lesion border, there were GFP+ cell processes crossing the GFAP defined border and into host tissue (Figure 3.7). These data suggest that the inclusion of AIMS does not inhibit the differentiation of pMNs into neuronal cell fates in vivo, and the neurons are capable of axonal extension that bridges into the host tissue providing the possibility of forming functional synaptic connections outside of the transplant area.

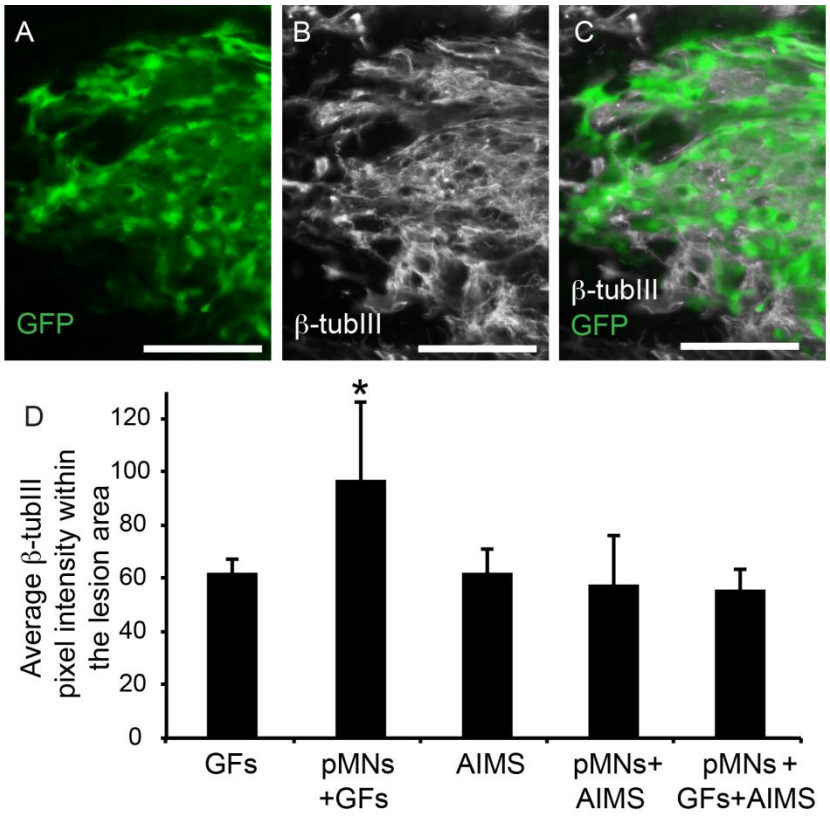

Figure 3.6: Spinal cord sections show colocalization of neuronal extensions and GFP$^+$ areas. (A-C) Representative images of GFP expression and a marker for activated neuronal extensions, β-tubIII, from GFs+pMNs group **(A)** and β-tubIII staining **(B)** that shows colocalization when merged together **(C)**. Robust β-tubIII staining was observed overlapping with GFP+ areas. **(D)** The average pixel intensity of β-tubIII staining in sections within the lesion area was determined. Error bars are standard deviation. Scale bar is 100 μm. * denotes significant difference from all other groups ($p<0.05$).

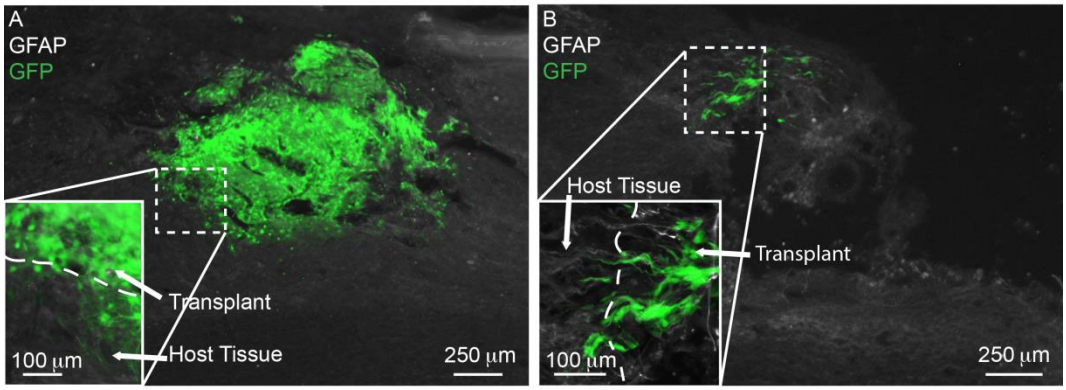

Figure 3.7: Penetration of transplanted pMNs into host tissue. Fluorescent GFP$^+$ images of injured spinal cord sections from **(A)** pMNs+GFs and **(B)** pMNs+GFs+AIMS were overlaid on top of the corresponding IHC images stained for the astrocyte specific marker (GFAP).

3.4.5 Fibrin Scaffolds with AIMS Decrease CSPG Deposition but have Limited Effects on CSPGs in Combination Scaffolds

Previous work in our lab and other lab's has shown that transplanting ChABC-loaded microtubes into the injured spinal cord reduces CSPG levels (Wilems-JCR submitted, (Meilander et al., 2001). The loaded ChABC remains active upon release from the microtubes and decreases CSPG deposition around the injury site in acute transplantation models. Transplantation of AIMS alone in the sub-acute injury significantly decreased CS56 staining, which marks non-degraded CSPGs, compared to groups containing pMNs+AIMS and pMNs+GFs+AIMS (Figure 3.8). However, the pMNs+AIMS group did not demonstrate a decrease in CSPG deposition within the lesion site despite the delivery of ChABC. The trends seen within the injury area were maintained in the immediate host tissue (within 250 μm of the injury), but differences in CS56 staining were not apparent more than 250 μm beyond the lesion (Figure 3.8F). Thus it appears the combination of pMNs+AIMS or pMNs+GFs+AIMS leads to a significant increase in CSPGs, which may inhibit axonal extension and the formation of synaptic connections within the transplant area and host tissue.

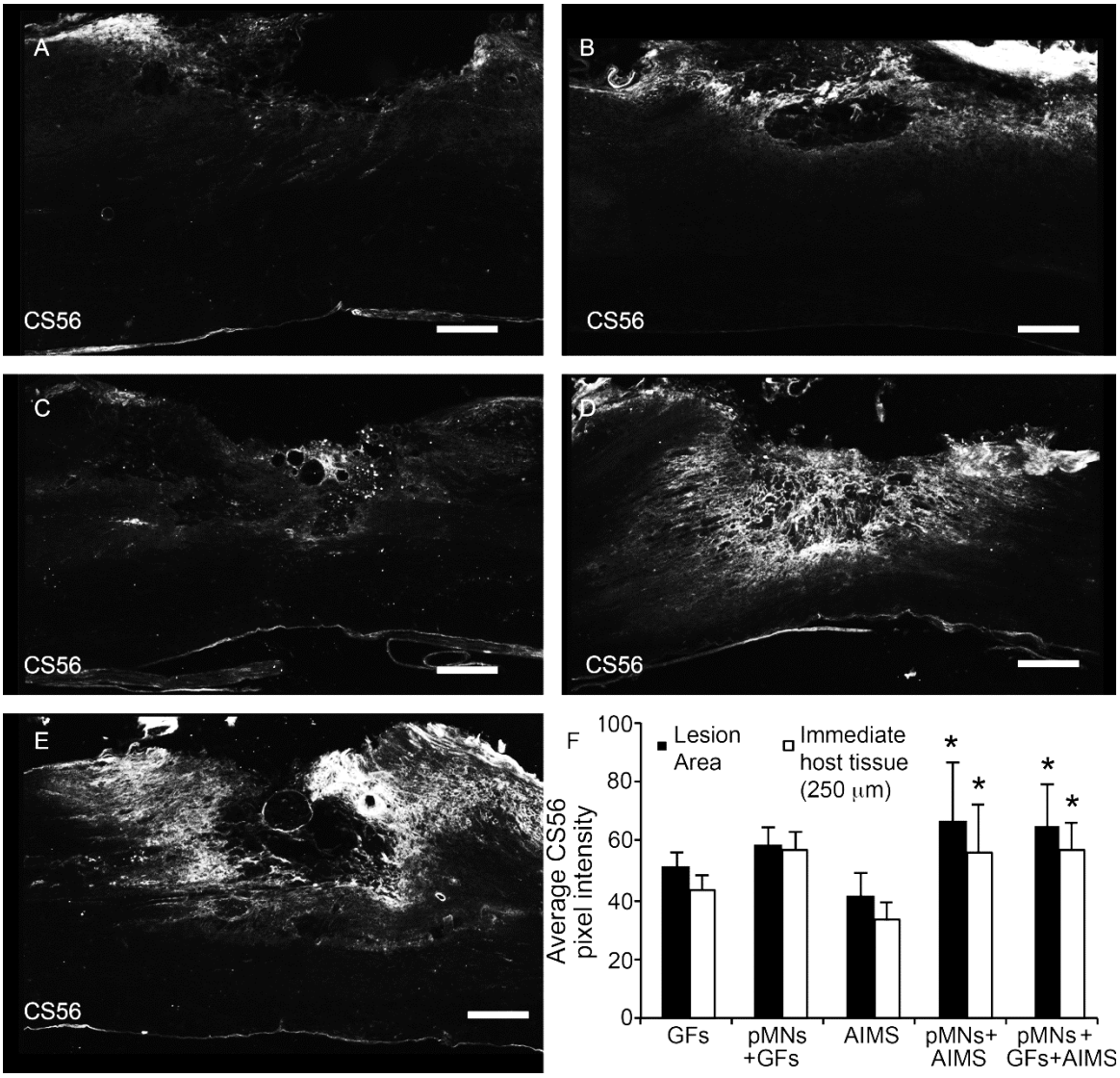

Figure 3.8: Combination of pMNs and/or GFs with AIMS does not decrease CSPGs compared to GFs alone or pMNs+GFs. (A-E) Representative images of CS56, a marker for non-degraded CSPGs, staining within and around the lesion site for groups containing (A) GFs, (B) pMNs+GFs, (C) AIMS, (D) pMNs+AIMS, (E) pMNs+GFs+AIMS. Strong CS56 staining was seen when pMNs and AIMS were included in fibrin scaffolds. **(F)** The average pixel intensity of CS56 stained sections within the lesion area and within the immediate host tissue (250 μm from the lesion border) was determined. Fibrin scaffolds with pMNs+AIMS or pMNs+GFs+AIMS had significantly higher ED1+ staining compared to the AIMS alone group in both the lesion area and immediate host tissue. Error bars are standard deviation. * denotes $p<0.05$ versus AIMS alone group

3.5 Discussion

As a proof of concept study, the drug delivery systems were incorporated into fibrin scaffolds and implanted into rats after SCI. A decrease in axonal extension was measured when unloaded microspheres and microtubes were transplanted within fibrin scaffolds by staining with antibodies against β-tubIII. This decrease may be caused by PLGA degradation byproducts which are acidic and lower the pH of the local environment (Sung et al., 2004). Acidic environments have been shown to affect the immunoreactivity of astrocytes for GFAP, which corresponds to astrocytes in a more reactive state (Oh et al., 1995). Therefore, the decreased pH caused by degradation of PLGA microspheres may drive astrocytes into a reactive state, as evidenced by an upregulation of GFAP. Astrocytes in a reactive state are detrimental to recovery because reactive astrocytes increase deposition of CSPGs and limit axonal extension; both an increase in GFAP and CSPG was measured with unloaded microspheres and microtubes. Therefore, the incorporation of PLGA microspheres into fibrin scaffolds may lead to increased CSPG deposition due to an enhanced conversion of astrocytes into reactive astrocytes and future research using the drug delivery systems should account for the potential negative effects on the local environment.

In contrast, the loaded drug delivery systems appeared to increase axonal extension and decrease the presence of CSPGs. Therefore, the sustained release of ChABC and NEP1-40 may increase axonal growth potential at the injury site by removing extrinsic barriers. The removal of extrinsic barriers including CSPGs and MAIs provide a more permissive environment leading to improved axonal extension. Sustained release of NEP1-40 and ChABC also had no effect on GFAP staining compared to the injury alone group, suggesting limited effect on astrocytes two weeks after injury. The inflammatory response after SCI is an important factor and acidic degradation byproducts PLGA may increase the inflammatory response (Kim et al., 2007). No significant

changes were measured in macrophage/microglia (ED-1) staining when PLGA microspheres and lipid microtubes were incorporated into fibrin scaffolds, although incorporation of microspheres and microtubes trended toward increased inflammation. This suggests that although the byproducts from the PLGA microspheres may increase macrophage infiltration and microglia activation, the increase was not significant when the microparticles were loaded with anti-inhibitory molecules and did not appear to limit neuronal growth at the site of injury. Future work using the microspheres and microtubes for drug delivery should monitor the local inflammatory response.

The secondary injury following SCI results in significant cell loss and formation of a complex, highly inhibitory glial scar. Many therapeutic options have targeted single obstacles that limit recovery following injury, which typically result in modest functional improvements. Here we combined several potentially promising therapies into a fibrin-based tissue engineered scaffold to overcome multiple obstacles simultaneously, with the aim of enhancing recovery over single therapies. Specifically, we aimed to repopulate the cystic cavity and limit the inhibitory effects found within the glial scar. Transplanting pMNs with the sustained delivery of neurotrophic factors verified previous work showing transplanted cells survived and differentiated into the expected cell types (McCreedy et al., 2014). The therapy incorporating AIMS into fibrin scaffolds also verified previous results by significantly decreasing the inhibitory CSPG presence around the lesion (Wilems – JCR Submitted). Interestingly, the combination of AIMS with pMNs did not result in an additive effect of the single therapies. Incorporating both AIMS and pMNs into fibrin scaffolds with or without GFs decreased cell survival and failed to reduce CSPGs around the lesion site.

The *in vitro* analysis of pMNs with AIMS did not show any adverse effects on cell viability at two weeks, which suggests that the AIMS only have a negative effect on pMN cell viability when transplanted *in vivo*. It is possible the AIMS indirectly limit survival through interactions with the

host environment; specifically, the AIMS may increase recruitment of inflammatory cells, such as macrophages, when combined with cell transplants. Inflammatory cells, including neutrophils and macrophages, generate oxygen free radical species and lysosomal enzymes that cause non-specific degradation of the surrounding cells and tissue (Cassatella, 1995, Taoka and Okajima, 2000). Scaffolds containing AIMS did not significantly increase macrophage infiltration compared to GFs alone. The lipid microtubes were not expected to affect pMN viability and were previously shown to have a limited effect on the inflammatory response *in vivo* (Meilander et al., 2001). The sugar trehalose, used for thermostabilization of ChABC within the lipid microtubes, has been shown to increase cellular resistance to oxidative stress by scavenging free radicals and could limit the cytotoxic effects within the lesion site thus improving cell survival (Benaroudj et al., 2001). In contrast to the lipid microtubes, hydrolysis of PLGA microspheres creates degradation byproducts that are acidic, lower the pH of the local environment, and have been shown to significantly affect cell viability in long term cultures (Sung et al., 2004). Furthermore, subcutaneous implantation of PLGA scaffolds into adult rats showed an enhanced inflammatory response compared to less acidic scaffolds (Kim et al., 2007). PLGA microspheres may increase long-term inflammation, but the AIMS incorporated into fibrin scaffolds without pMNs did not trigger increased macrophage infiltration. It is likely that individually, AIMS and pMNs both affect infiltration and activation of inflammatory cells, but the inflammation is limited until the two therapies are combined.

After transplantation, P-Olig2-derived pMNs retain high GFP expression, which colocalized to astrocyte (GFAP) and neuronal markers (NeuN), though oligodendrocyte (O4) staining was inconclusive. Importantly, the average density of $NeuN^+$ cells per GFP^+ area was consistent across all groups containing pMNs. The neuronal markers appeared within GFP^+ areas in much higher quantities compared to astrocyte markers. GFAP has been shown to be highly upregulated within reactive astrocytes, but has lower levels of expression in more growth permissive astrocytes

(Bardehle et al., 2013, Pekny et al., 2014). Therefore, the quantity of astrocytes may be higher than the GFAP staining suggests, and the astrocytes within the transplant area may be more growth permissive and less reactive than the host astrocytes located at the injury border. Furthermore, the transplanted neurons extended robust axonal projections within the transplant area, bridged the injury site, and extended processes into the adjacent host tissue, which could enable synaptic connections between host and transplanted cells and possibly lead to functional circuits. Transplanted pMNs are capable of differentiating into the appropriate cell fates in the presence of AIMs, demonstrating the feasibility of the combined system; and AIMS-induced inhibition to differentiation did not pose a significant obstacle.

Lastly, the combination of AIMS and pMNs within fibrin scaffolds appears to have limited the effect of ChABC delivery from lipid microtubes into the lesion site. Active ChABC was expected to degrade CSPGs in the local tissue, but became either inactive or phagocytosed prior to diffusing beyond the transplant area. The transplanted cells or the increased macrophage/microglia presence within the injury site may have been responsible for the loss in ChABC bioactivity. While the ChABC was able to reach deposited CSPGs when AIMS were transplanted alone, the combination of AIMs and pMNs actually resulted in higher amounts of CSPGs within the transplant area and in the surrounding host tissue than with either treatment alone. The higher CSPG deposition may also be a result of increased reactive gliosis. PLGA degradation byproducts can decrease the local pH, which has been shown to direct astrocytes into a reactive state (Oh et al., 1995).

3.6 Conclusion

In conclusion, research has resulted in many promising therapies that aim to overcome specific obstacles limiting recovery. Our lab has previously developed several therapeutic options that allow for cell transplantation of highly purified pMNs, the sustained delivery of neurotrophic factors, or the sustained delivery of ChABC and NEP1-40. In this study, we combined the therapeutic strategies within fibrin scaffolds with the aim of improving recovery over individual treatments. All treatments containing pMNs had viable cells two weeks post-transplantation into the injured spinal cord, although the incorporation of AIMS with pMNS led to a decrease in cell survival, possibly due to an enhanced inflammatory response within the lesion site. Surviving cells were capable of differentiating into neural cell fates, extending axons, and migrating into the host tissue. Treatment options with AIMS in fibrin scaffolds showed a decreased presence of CSPGs, but this decrease was lost when AIMS and pMNs were combined. Future work will be improving the AIMS by decreasing their effect on pMN survival and limiting their effect on the inflammatory response. Studying the long term effects of the combination therapies on functional recovery is important to understanding how the transplants are incorporating into the host tissue.

Chapter 4

Summary of Findings and Future Directions

4.1 Summary of Findings

Throughout this work we have strived to emphasize the complexity that is inherent with spinal cord trauma and the follow up treatment. Previous work in our lab and the second chapter in this thesis have produced therapies for treating SCI. Specifically, Dr. Phillip Johnson and Dr. Stephanie Willerth studied the effects of controlled growth factor delivery on mouse ESC-derived neural progenitors within fibrin scaffolds both *in vitro* and *in vivo*. This work paved the way for Dr. Dylan McCreedy to improve upon the therapy by using highly purified pMNs through collaboration with Dr. Cara Rieger from Dr. David Gottlieb's lab. Dr. McCreedy was able to demonstrate the survival and differentiation of the selected transgenic pMNs (p-Olig2 line) in a sub-acute dorsal hemisection model of SCI, while also removing the tumorgenic potential of the transplants compared to unpurified ESC-derived neural progenitors. The thesis presented here looked to further improve on the therapy by incorporating the sustained delivery of anti-inhibitory molecules into the fibrin scaffolds. The development and fabrication of the anti-inhibitory microparticle systems (AIMS) was accomplished in the first part of this work. The second part of was designed to show the efficacy of transplanting the AIMS into an acute rat SCI model, and to evaluate the effect a combination therapy of AIMS, pMNs, and sustained growth factor delivery had on the local environment after transplantation in a sub-acute injury model.

The first aim was to develop and fabricate the drug delivery systems capable of sustained release of NEP1-40 and ChABC. The delivery of the two molecules was achieved using two different drug delivery systems. The highly labile ChABC enzyme was thermally stabilized using trehalose and was loaded into hollow cylindrical lipid microtubes that had been dried. Loading occurred through capillary action and release from the microtubes was based on diffusion of the enzyme from the microtubes and out of the fibrin scaffolds. The small peptide, NEP1-40, was loaded into PLGA microspheres using a water in oil in water double emulsion evaporation technique. The release rate of NEP1-40 is directly dependent upon the degradation rate of the PLGA microspheres, and the formulation parameters chosen allowed for a relatively fast release rate due to the ratio of lactide to glycolide monomers and the intrinsic viscosity of the polymers. Both AIMS were shown to provide sustained active release of the anti-inhibitory molecules *in vitro,* and then the AIMS were transitioned into *in vivo* rat SCI models.

The second part of this work not only tested the efficacy of using AIMS to treat SCI but also combined the AIMS with the fibrin scaffolds. The efficacy of using AIMS to treat SCI was accomplished by incorporating the AIMS into fibrin scaffolds and transplanting the scaffolds into a rat dorsal hemisection immediately after injury. The treatment with AIMS was shown to increase axon extension and limit the presence of CSPGs two weeks after transplantation, although a trend to increased inflammation and reactive gliosis was seen compared to the injury alone group. The combination therapeutic strategy incorporating highly purified pMNs, the sustained delivery of neurotrophic factors, and the sustained delivery of ChABC and NEP1-40 into fibrin scaffolds produced very interesting results. Fibrin scaffolds containing pMNs and AIMS did not show decreased cell survival when cultured for two weeks *in vitro*. However the incorporation of AIMS with pMNs in a sub-acute rat SCI model led to a decrease in cell survival, which I believe was due to an increased inflammatory response when both AIMS and pMNs are present. Transplanted cells

that survived up to 2 weeks were capable of differentiating into neurons, extending axons, and migrating into the host tissue independent of the inclusion of AIMS. Treatment with AIMS in fibrin scaffolds alone showed a decreased presence of CSPGs but this effect was lost when AIMS and pMNs were combined.

In conclusion, I tried to improve upon the scaffolds previously established in the lab by introducing a system that removes the inhibitory cues found within the extracellular environment. I was able to develop this system through the use of PLGA microspheres and lipid microtubes with positive results when transplanted into an acute SCI model. However problems with cell viability and increased macrophage infiltration arose when AIMS were combined with cell transplantation in fibrin scaffolds. Future work should look into improving the AIMS, alternative anti-inhibitory molecules, and performing long term animal studies to better elucidate the effect the therapies have on functional recovery.

4.2 Future Directions

4.2.1 Improving the AIMS

The use of lipid microtubes to provide sustained delivery of ChABC was shown to significantly decrease the presence of CSPGs but there are several other options for sustained delivery of the enzyme. Research has achieved delivery of the highly labile ChABC enzyme through natural and synthetic drug delivery systems, although I do not believe most of these options are superior to the lipid microtubes (Huang et al., 2011, Liu et al., 2012, Rossi et al., 2012, Pakulska et al., 2013, Bartus et al., 2014). A recent publication used a lentiviral vector to transfect cells to

continually express ChABC following injury and showed improved functional recovery, axonal conduction, and serotonergic innervation (Bartus et al., 2014). This treatment is promising but has limitations due to the use of lentiviral vectors, which continually express the enzyme. I believe that the lipid microtube system could be replaced by transfecting the cells with the ChABC enzyme used by Bartus *et al.* but instead of the ubiquitously expressed promoter use an inducible promoter system that provides temporal control of expression.

The use of PLGA microspheres to deliver NEP1-40 may be the reason for the enhanced inflammatory response and should either be replaced as a drug delivery system, or the system should be modified to mitigate inflammation. I am not aware of any publications that provide an alternative method to sustained local drug delivery of the peptide without the use of intrathecal pumps, although a publication has used systemic treatment options including intraperitoneal injection and subcutaneous osmotic pumps with both showing improved axonal sprouting rostral to SCI (Li and Strittmatter, 2003). Moving to a systemic delivery method may eliminate the need for the PLGA microspheres and decrease the amount of material being placed within the fibrin scaffolds.

If the AIMS continued to be used, I recommend a change in the transplantation protocol. In the studies performed within this thesis, the AIMS and pMNs were combined into a single fibrin scaffold, placed into the injury cavity, and a second fibrin scaffold containing no additional factors was placed on top of the first scaffold. If the pMNs were instead included in one scaffold, and the AIMS were included in a second scaffold, the cell viability may be increased. The released anti-inhibitory molecules could still be delivered to the injured cord, but the diffusion distance to their targets may be significantly increased. This also assumes that the inflammation would be decreased by separating the AIMS and pMNs, which would need further validation using animal models.

4.2.2 Long Term Studies to Evaluate Functional Recovery

Independent of the changes made to the AIMS and direction that this research takes, the need for long term studies in SCI is important. The main goal of all therapies in SCI is to re-form functional synaptic connections that will improve functional recovery. For example, when the AIMS were used in the acute SCI model, an increase in axonal extension was measured. Long term studies would allow us to determine if the axons were capable of forming meaningful connections that promote motor recovery. Furthermore, the highly purified pMNs have now been used in 2 sub-acute injury models and showed high cell viability, differentiation into neurons, robust extension of axons, and migration into the host tissue. This suggests that the pMNs may significantly affect recovery due to the potential to form synaptic connections over and through the transplant area but long term functional studies are required to further evaluate the therapeutic potential of the cells.

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Vita

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EDUCATION

Washington University in St. Louis, Saint Louis, Mo

Ph.D., Biomedical Engineering — August 2015

Texas A&M University, College Station, Tx

B.S., Biomedical Engineering / Tissue-Engineering Tract — May 2010

RESEARCH EXPERIENCE

Washington University in St. Louis, Biomedical Engineering — 2010 - 2015

Doctoral Research Student, Mentor: Prof. Shelly Sakiyama-Elbert, Ph.D.

Thesis: The Development of Drug Delivery Systems for use in Spinal Cord Injury

- Developed and modified synthetic and natural biomaterials for controlled temporal delivery of anti-inhibitory drugs, neurotrophic growth factors, and stem cell derived neural progenitors for use in a rat thoracic spinal cord injury model.

Texas A&M University, Biomedical Engineering — 2007 - 2010

Undergraduate Research Assistant, Mentor: Prof. Elizabeth Cosgriff-Hernandez, Ph.D.

- Synthesis of novel block copolymers. Development of biodegradable polyurethane scaffolds to aid in ligament repair. Main focus was establishing synthesis protocols and optimizing the synthesis route of the scaffolds.
- Synthesis of novel poly(ethylene glycol) bioactive hydrogels based on designer collagens. Main focus was to synthesize a library of bioactive hydrogels to later be used to identify a set of vascular graft formulations.

AWARDS/ORGANIZATIONS

- National Science Foundation Graduate Research Fellow 2011-2014
- BALSA Consulting Group – Consultant 2014
- Graduate Student Advisory Board Representative 2014
- ISNR Student Travel Award Winner 2013
- Poster Award Winner at Texas A&M University's Biomaterials Day 2010
- (NSF) Louis Stokes Alliance for Minority Participation Grant Recipient 2008, 2009
- Undergraduate Research Scholarship Recipient 2009
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- Biomedical Engineering Honor Society (Alpha Eta Mu Beta) Member 2009-2010

PUBLICATIONS IN PRINT

1. McCreedy D, **Wilems T**, Xu H, Butts J, Brown C, Smith A, Sakiyama-Elbert SE. "Survival, Differentiation, and Migration of High-Purity Mouse Embryonic Stem Cell-derived Progenitor Motor Neurons in Fibrin Scaffolds after Sub-Acute Spinal Cord Injury," *Biomaterials Science* 2: 1672 - 1682, 2014.
2. M.B. Browning, **T. Wilems**, E. M. Cosgriff-Hernandez, "Compositional control of poly(ethylene glycol) hydrogel modulus independent of mesh size," *J Biomed Mater Res*, 98A, 268-273 (2011).
3. E. M. Cosgriff-Hernandez, M.Hahn, B.Russell, **T.Wilems**, D. Munoz-Pinto, M.B. Browning, J. Rivera, M. Hook, "Bioactive Hydrogels Based on Designer Collagens," *Acta Biomaterialia*, 6, 3969-3977 (2010).

PUBLICATIONS IN PREPARATION

1. **Wilems T**, Sakiyama-Elbert SE. "Sustained Dual Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury Treatment." 2015.
2. **Wilems T,** Pardieck J, Iyer N, Sakiyama-Elber SE. "Combination Therapy of Stem Cell Derived Neural Progenitors and Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury." 2015.

CONFERENCE PRESENTATIONS

Oral Presentations

1. **Wilems T,** Sakiyama-Elbert, "Combination Therapy of Stem Cell Derived Neural Progenitors and Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury." Society for Biomaterials Meeting, April 2015.

2. **Wilems T**, Ingram, Sakiyama-Elbert, "Sustained *In Vivo* Dual Drug Delivery of Anti-Inhibitory Molecules for Spinal Cord Injury Treatment." Biomedical Engineering Society Meeting, October 2014.
3. **Wilems T [Presenting Author]**, Browning, Hahn, Munoz-Pinto, Rivera, Höök, Russell, Cosgriff-Hernandez, "Incorporation of Collagen-Mimetic Proteins into Bioactive Hydrogels." Society for Biomaterials Meeting, April 2010.

Poster Presentations

1. **Wilems T [Presenting Author]**, McCreedy, Marquardt, and Sakiyama-Elbert, "Poly(lactic acid-co-glycolic acid) Microspheres for Sustained Delivery of Bioactive Molecules for Spinal Cord Injury Repair." Society for Biomaterials Meeting, April 2013.
2. **Wilems T [Presenting Author]**, Ingram, Sakiyama-Elbert, "Development of biomaterials for sustained delivery of bioactive molecules in spinal cord injury." International Symposium for Neural Regeneration, December 2013.
3. Browning, **Wilems T [Presenting Author]**, Hahn, Cosgriff-Hernandez, "Decoupling PEG Hydrogel Mesh Size and Modulus with the Integration of 4-armed PEG." Society for Biomaterials Meeting, April 2010.
4. Munoz-Pinto, Wang, **Wilems T**, Browning, Cosgriff-Hernandez, Russell, Rivera, Höök, Hahn "Bioactive Hydrogels based on Collagen Mimetic Proteins for Controlled MSC Differentiation." Society for Biomaterials Meeting, April 2010.
5. Benhardt, **Wilems T**, Cosgriff-Hernandez, "Cell-Responsive Polyurethanes: Synthesis of Peptide-Based Polyol Soft Segments." Society for Biomaterials Meeting, April 2009.
6. Cosgriff-Hernandez, **Wilems T [Presenting Author]**, Sears, "Peptide-based Polyurethane Elastomers as Tissue Engineering Scaffolds." Materials Research Society Meeting, November 2008.