UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Templated Agarose Scaffold for Axon Guidance in the Central and Peripheral Nervous System

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Thomas Richard Gros

Committee in charge:

Professor Mark H. Tuszynsksi, Chair
Professor Gabe A. Silva, Co-Chair
Professor Armin Blesch
Professor David A. Gough
Professor Xiaohua Huang

2009

The Dissertation of Thomas Richard Gros is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009

This thesis is dedicated to:

my family, that nurtured and supported my ambitions

my friends, that inspired and guided my dreams

and my love, who gave me perspective and hope for the future.

Everything should be made as simple as possible, but no simpler.

Albert Einstein

Imagination is more important than knowledge.

Albert Einstein

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

Acknowledgments

I'd like to thank my family that nurtured and supported my ambitions, my friends that inspired and guided my dreams, and my love, who gave me perspective and hope for the future.

Chapter 2 is original work in preparation as Gros TR, Sakamoto J, Blesch A, Havton L, Tuszynsksi MH. Templated agarose scaffolds orient and guide regenerating long-tract axons through sites of spinal cord injury and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

Chapter 3 is original work in preparation as Gros TR, Sakamoto J, Blesch A, Tuszynsksi MH. A physical and chemical combinatorial therapy enhances descending axon guidance across a complete spinal cord transection and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

Chapter 4 is original work in preparation as Gros TR, Sakamoto J, Tuszynsksi MH. A highly linearized and mechanically scalable hydrogel nerve conduit for peripheral nerve regeneration and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

Vita and Publications

2002 B.S., Chemical Engineering, University of California, San Diego

2005 M.S., Neurosciences, University of California, San Diego

2009 Ph.D., Neurosciences, University of California, San Diego

Publications

Gros TR (2003) The Gros similtude equation for scaling up batch reactors of non-newtonian fluids. Gen-Probe internal document.

Patent # **60/967,091**, (2008) Co-inventor. Scaled-up nerve guidance scaffold technology for central and peripheral nerve repair.

Gros TR, Sakamoto J, Blesch A, Tuszynski MH (2009) Templated Agarose Scaffolds Orient and Guide Regenerating Long-Tract Axons Through Sites of Spinal Cord Injury.

Awards

2005-2009	National Institute of Health Graduate Research Fellowship
2005	Bioengineering TA of the Year
2003	Gen-Probe Silver Pen Award- Early Completion of West Nile Virus Test Kits
2002	Graduated Summa Cum Laude, Provost Honors 8X, Phi Beta Kappa Honors Society, UCSD

ABSTRACT OF THE DISSERTATION

A Templated Agarose Scaffold for Axon Guidance in the

Central and Peripheral Nervous System

by

Thomas Richard Gros

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2009

Professor Mark H. Tuszynski, Chair

Professor Gabe A. Silva, Co-Chair

This thesis examined the hypothesis that axonal guidance could be improved in the central and peripheral nervous systems using a highly linearized templated agarose scaffold. In the present study we examined whether a templated agarose scaffold improved axon retention across a large central nervous system (CNS) lesion and how cellular and axonal orientation was affected within the scaffold channels. The "physical" guidance from the scaffold was applied to an existing CNS "chemical" guidance strategy, shown to promote axons *beyond* the lesion site, to enhance the number of crossing axons in larger, disorganized, lesions. Specifically, there was the greatest number of long-tract sensory axons reaching the distal aspect of the lesion when the templated agarose scaffold was combined with a neurotrophic source of NT-3 beyond the lesion site and a conditioning lesion, to enhance chemical axon guidance and the intrinsic growth state of axons, respectively. When comparing the scaffold

implant to a cell suspension grafts, we found a higher retention of long-tract ascending (sensory) axons and descending (motor) axons crossing large lesions (2mm). The enhanced axon retention may be attributed to the finding that cellular orientation within the scaffold channels is highly linear, thus promoting a less tortuous environment for axon orientation and bridging. Although an enhanced number of axons were able to cross the lesion, the axons did not repenetrate the host tissue due to a reactive cell layer, present only in scaffold the implant groups. Additionally, a peripheral nerve conduit, with the agarose scaffold as the core, displayed biocompatiablility and supported axon growth and vasculature beyond the clinically applicable distance of 4mm. Thus, the templated agarose scaffold enhances axon retention and guidance within CNS injury sites and has potential applications to the PNS.

CHAPTER 1

Introduction

Spinal cord injury overview

Spinal cord injury is a devastating life-long disability affecting nearly 250,000 Americans, with approximately 11,000 new injuries a year. Approximately, 52% of injuries are considered paraplegic and 47% quadriplegic. In contrast to many other debilitating medical problems, the mean age of spinal cord injury is 31, a population with a high socio/economic impact. Spinal cord injuries most often occur in vehicular accidents (37%), violence (28%), falls (21%), and sport-related accidents (6%) ("SCI Info", 2008).

To regain function in spinal cord injury, nerve regeneration is necessary. Regeneration is a complex process that must overcome many obstacles. Although many advances have been made in the field to address individual aspects of spinal cord injury, it is becoming increasingly clear that a combinatorial therapy addressing multiple mechanisms of injury is necessary. Furthermore, to address these multiple obstacles of spinal cord injury, it is likely that we must first understand the three stages of spinal cord injury; acute, secondary, and chronic stages. Briefly, the acute stage of spinal cord injury, caused from the initial impact, results in immediate transection and contusion of axons (Rosyn et al., 2008). Often the fractured vertebrate insults the site of injury, creating inflammation and swelling. Furthermore, cell death

occurs from ischemia, hemorrhage, edema, and electrolyte shits (McDonald et al., 2002). During the secondary injury stage, which occurs from days to weeks, there is formation of a cystic cavity that often expands beyond the site of initial impact. An inhibitory scar consisting of local astrocytes is formed along the lesion cavity, known as reactive gliosis, and inhibitory molecules are released (Tator, 1995). In the chronic stage of injury, occurring over weeks to years, there is further deposition of inhibitory molecules, such as proteoglycans, and axon demyelination (Rosyn et al., 2008).

Clinically, methods to reduce secondary inflammation are implemented. Mechanical methods include a laminectomy that can be performed to reduce swelling and ischemia. Additionally, orthopedic methods are used to stabilize bone fragments from entering and further damaging the injury site (Fehlings et al., 2001). Chemically, steroids such as methylprednisolone, have been used to reduce inflammation (Short et al., 2000). Although these treatments are commonly available, they mainly serve as a means to prevent further damage to the injury than addressing spinal cord repair. Clearly, there is a need for further development in many aspects of spinal cord repair. Following is a summary of the current understanding of specific aspects of spinal cord injury and a review of the respective research paradigms for spinal cord repair.

Challenges of spinal cord injury

Inhibition of axon growth in glial scar

An inhibitory glial scar often forms within the first two weeks of injury (Berry et al. 1983). During the first few days of scar formation, macrophages migrate from the circulation into the lesion site to remove cellular debris (David et al. 1990). Next, meningeal cells and oligodendrocyte precursors invade the injury site to isolate the host parenchyma from potentially harmful effects of the external environment, reducing hemorrhaging, ischemia, edema and electrolyte shifts (Levine et al. 2001). Near the end of the two weeks, cells thought to be the predominant source of molecular inhibition invade the injury site. Specifically, reactive astrocytes and oligodendrocytes invade the injury cite, releasing a milieu of inhibitory molecules (McDonald et al., 2002; Jones et al. 2002).

Axon regeneration is thought to be inhibited through the spinal cord injury site due to the presence of molecules formed by the reactive extracellular matrix, the glial scar, and myelin inhibition (Liu et al., 2006; Zhang et al., 2006). Molecular inhibitors are thought to inactivate the substrate and receptors which signal axon mobility. Specifically, a strongly charged extracellular matrix molecule glycosaminoglycan (GAG) side chain is though to inactivate the matrix and molecules responsible for axon extension (Bradbury et al., 2002). During different times of scar development, oligodendrocytes precursors, meningeal cells, and astrocytes are all though to release inhibitory molecules. Chondroitin sulfate proteoglycans (CSPGs) are thought to be

the predominate inhibitory molecules released from reactive glial. The presence of CSPGs have been confirmed in the glial scar in many spinal cord injury models (Fitch and Silver, 1997; Rhodes et al., 2004). Furthermore, it has been shown that CSPG expression creates a non permissive growth environment to deter axon growth during injury and developmental stages (Fitch and Silver, 1997; Grimpe and Silver, 2004).

Reactive astrocytes within the glial scar have been shown to release molecules such as tenascin (Apostolova et al., 2006), semaphorin-3 (Pasterkamp et al., 2001), and chondroitin sulfate proteoglycans such as neurocan and phosphacan (McKeon et al., 1999; Rhodes and Fawcett, 2004; Jones et al., 2003). Some of these specific molecules released by the reactive astrocytes have been proven to be inhibitory to long distance axon growth in *in vitro* (Ness and David, 1997; Tom et al., 2004), and in *in vivo* models (Houle et al., 2006; Steinmetz et al., 2005). Additionally, oligodendrocytes have been also shown to express CSPGs such as NG2 and neurocan made by oligodendrocyte precursors (Chen et al., 2002, Levine et al. 2001). Furthermore, meningeal cells have been shown to produce inhibitory molecules such as tenascins, proteoglycans, and semaphorins (Zhang et al. 1997; Pasterkamp et al. 1999; Niclou et al., 2003). Interestingly, meningeal cells are thought to aid in the formation of the inhibitory astrocytic scar (Shearer and Fawcett, 2001). Thus, methods to overcome the glial scar may include promoting axon growth through the lesion and blocking the inhibitory molecules themselves.

Downregulation of neuronal support in CNS

Neurotrophic factors have long been thought to be transported down the axon to promote neuronal survival during development (Korsching, 1993). Axonal growth cones appear to be partially guided by chemoattraction, from growth factors (Goodman, 1996; Gallo et al., 1997; Song and Poo, 1999). Specifically, growth factors are thought to guide developing axons (Behar et al., 1997; Tucker et al., 2001; Fritzsch et al., 1997). Studies support the concept that when a neuron is injured, neurotrophic support is needed to maintain cell survival and promote neurite outgrowth (Giehl et al., 1998; Meiri et al., 1998; Giehl and Tetzlaff, 1996).

Unlike the peripheral nervous system (PNS), the injured neurons generally do not express these growth factors themselves in an autocrine and paracrine manner. However, direct application of neurotrophins to a spinal cord injury have been shown to rescue neurons and promote axon growth (Jones et al., 2001; Blesch et al., 2002). Furthermore, it has been shown that growth factor gradients have been able to enhance axon regeneration beyond a site of a spinal cord lesion (Taylor et al., 2006). Therefore, neurotrophic support may be a means to support neurons and promote axon guidance across and beyond a lesion site, allowing them to potentially reconnect to their targets, and restore function to spinal cord injury.

Downregulation of regeneration associated genes in CNS

Although neurons showed an intrinsic capacity to regenerate in the peripheral nervous system (PNS) (Cajal, 1991; Richardson et al., 1980), their regenerative

capacity was reportedly reduced in the CNS due to altered expression of regenerative association genes (RAGs) (Schreyer and Skene, 1993; Broude et al., 1997). However, it is thought that when the RAG gene expression is enhanced, axons are able to grow through the inhibitory injury site (Richardson and Issa, 1984; Neumann and Woolf, 1999; Leon et al., 2000).

Specifically, the GAP-43 gene was shown to regulate the actin cytoskeleton (Frey et al., 2000), necessary for axon extension on substrates such as N-CAM (Meiri et al., 1998). In a model, zebrafish, innately capable of regenerating central nervous system axons, gene expression profiles showed an upregulation of GAP-43 and other regeneration associated genes in neurons that extended axons into the inhibitory central nervous system lesion (Becker et al., 1998). Additionally, it is thought that axons in a peripheral nerve injury are more capable of regeneration than axons in a spinal cord injury due to upregulation of regeneration associated genes, such as GAP-43 (Madsen et al., 1998). However, in mice models, genetic overexpression of single growth associated genes, through viral delivery, did not show enhanced neurite outgrowth into the central nervous system lesion (Bomze et al., 2001). Therefore, it is likely that simultaneous upregulation of multiple regeneration associated genes are needed to enhance the intrinsic growth state of central nervous system neurons.

Lesion cavity formation

After server spinal cord injury, lesions often result in cystic cavities. Cells migrate into the lesion site to wall off the lesion boundary, replacing the function of

the dura, and effectively mediating additional damage from hemoraging, ischemia, and edema (Tator et al., 1995). However, this often causes a formation of a large fluid fill cyst within the lesion cavity, which is not permissive to axonal extension (Tator et al.,1995; Quencer et al., 1996). The fluid filled cavity does not serve as a substrate physically capable of supporting axon regeneration, as axon extension often need a substrate with a greater density than CNS fluid (Tuszynksi and Kordower, 1999).

Cellular grafts are often used to fill the lesion cavity after a spinal cord injury with the intention of providing a permissive environment (Ronsysn et al., 2008). Often cellular grafts are implanted with the aim to either mimic (embryonic stem cells), repair (Schwann cells), or enhance (genetically modified fibroblasts) the host environment to support axon regeneration. Axonal attachment and elongation through cellular grafts has been observed in fibroblasts (Pizzi and Crowe, 2006; Jin et al., 2002), astrocytes (Wang et al., 1995), Schwann cells (Franklin et al., 1996), olfactory ensheathing cells (Lu et al., 2002) and bone marrow stromal cells (Chopp et al., 2000). However, in large lesions, the cellular grafts often display a disorganized cellular architecture, resulting in a minimal amount of regenerating axons reaching the distal aspect of the spinal cord (Lu et al., 2005).

Approaches to enhance spinal cord regeneration

Blocking glial scar inhibitory molecules

As described above, there is a milieu of inhibitory molecules released from the glial scar. Methods to reduce the inhibition created from the scar often involve attempts to block or remove the inhibitory properties of each molecule (Silver, 1994). Although the list of inhibitory molecules within the glial scar is growing there has been some success in blocking the predominant inhibitory molecules, chondroitin sulfate proteoglycans (Grimpe and Silver, 2004). Methods to degrade the negatively charged GAG side chains with chondroitinase resulted in an increase in axon regeneration (Yick et al., 2000; Barritt et al., 2006; Bradbury et al. 2002). Additionally, it has been shown that by removing a dominant source of the inhibitory CSPG's, reactive astrocytes, there is an increase in axon regeneration (Bush et al., 1999). However, some molecular GAG side chains, such as those on NG2, are not fully inactivated by the chondroitinase (Dou et al., 1994). Although there have been many studies attempting to block the inhibitory molecules, there is a growing list of additional inhibitory molecules which remain unchecked. Thus, there is likely a need to enhance the axon's ability to overcome the inhibitory environment within the glial scar.

Growth factor delivery

As previously stated, growth factors are thought to guide developing axons (Behar and Barrett, 1997; Tucker et al., 2001; Fritzsch et al., 1997) and enhance survival and growth of injured neurons (Giehl et al., 1998; Meiri et al., 1998; Giehl and Tetzlaff, 1996). Thus, growth factor delivery may provide a means to promote axon extension into and possibly beyond spinal cord injury site (Jones et al., 2001). Previously growth factor delivery has been administered via direct injections, osmotic pumps, and saturated scaffolds to enhance axon growth (Schnell et al., 1994; Bradbury et al., 1999; Shibayama et al., 1998). However, using these methods, growth factor delivery is not specifically localized to the region of interest over long periods of time, which is potentially necessary to elicit a robust regeneration of axon growth and further promote axons beyond the site of injury. Therefore, genetic therapies have been developed to deliver growth factors in a long-term, localized gradient to the lesion site (Taylor et al., 2006). Additionally, cells have been genetically modified *in vitro* and then implanted into the spinal cord lesion to deliver a relatively sustained release of growth factors. Specifically, fibroblasts, Schwann cells and bone marrow stromal cells have previously been modified to release growth factors into the injury site with some success of enhancing axonal regeneration (Nakahara et al., 1996; Menei et al., 1998; Lu et al., 2005). Furthermore, a growth factor gradient was created by implanting genetically modified cells into a lesion site and administering a growth factor gene therapy *beyond* the injury site, resulting in axon growth beyond the lesion site (Taylor et al., 2006). Thus, by mimicking the growth factor gradients during

development, direct gene therapy and/or genetically enhanced implanted cells may enhance axon regeneration by providing bioactive growth factor, in a sustained release, within a spatially controlled manner.

Enhance neuronal intrinsic growth capacity

Central nervous system axons do not readily regenerate into sites of spinal cord injury, while they do in peripheral nerve injury. However, nearly 100 years ago, Cajal proposed that chemical agents from the distal nerve stump in the peripheral nerve injury could attract the regenerating proximal axons (Ramon y Cajal, 1928). Later, it was confirmed that if axons in peripheral nerves were previously cut ("conditioning lesion"), axons extending from these dorsal root ganglia neurons were capable of regenerating into the spinal cord (Richardson and Issa, 1984; Neuman and Woolf, 1999). It has been shown that the peripheral nervous system upregulates many regeneration associated genes after injury to promote axon regeneration that the central nervous system does not (Fernandes and Tetzlaff, 2000). Thus, the "conditioning lesion" created from injured periphery neurons may have promoted their central projecting axons to regenerate into the spinal cord lesion.

Specifically, it is thought that the conditioning lesion upregulates regeneration associated genes to enhance the intrinsic growth state of sensory axon populations extending into the central nervous system (Plunet et al., 2002). Growth associated protein, such as GAP-43, were shown to be downregulated with a spinal cord lesion, but upregulated when a conditioning lesion was applied (Fernandes et al.,2000;

Vaudano et al., 1995; Campbell et al., 1991). Although studies have used gene therapy to upregulate the growth associated genes in the central nervous system (Buffo et al., 1997), they did not result in significant enhancement of axon regeneration into the spinal cord lesion site. Alternate methods to upregulate regeneration associated genes, such as cAMP delivery, have been administered with some success (Qiu et al., 2002; Neumann et al., 2002). Furthermore, methods to combine the enhanced intrinsic growth state with other growth therapy methods have been studied (Lu et al., 2004). However, methods to delivery extrinsic treatments to enhance the intrinsic growth state remain inconsistent. Therefore, methods such as a conditioning lesion will serve as an effective and reproducible method to increase the intrinsic growth state of regenerating sensory axons into the site of spinal cord injury.

Bridge lesion cavity

A cystic cavity often forms at the site of spinal cord injury, filling with a fluid which axons are unable to regenerate across (Tuszynski and Kordower, 1991). In many current research paradigms, cellular therapies are used to fill a lesion cavity and release biological factors, thus providing a substrate for axon regeneration to occur (Lindval et a., 2004; Jones et al., 2001; Vroeman et al., 2003; Takami et al., 2002; Lu et al., 2005). However, cell survival is low, and biological factor can be diminished in large lesions (Friedman et al., 2002). Thus, if the injury is too large, a material scaffold may be used to enhance implanted cell survival and further control release of biological factors to the specific target area (Nomura et al., 2006). Therefore, a

biocompatible scaffold has the potential to promote axon growth across large spinal cord lesions.

In vitro axonal guidance has been enhanced by the physical architecture of substrates. *In vitro* axon guidance has been shown to be enhanced along micro-grooves within the growth substrate (Clark et al., 1991; Clark et al., 1992; Clark et al., 1993). Specifically, *in vitro* neurite alignment was enhanced in the approximate upper range of 10-40*u*m, which is similar to cellular diameters (Miller et al., 2002; Houchin-Ray et al., 2007; Shaw and Shoichet, 2003). Additionally, the lower limit of physical guidance of *in vitro* axons was 2*u*m (Ito, 1999), similar to axon bundle sizes.

In vivo studies with controlled micro and macro structures have shown some signs of improvement to sites of spinal cord injury (Moore et al., 2006; Yan et al., 2005; Teng et al., 2002). Even, empty tubes have been shown to enhance formation of tissue bridges (Tsai et al., 2004), likely reducing the lesion cavity size. Increasing the surface area of the scaffold inner scaffold interface showed enhanced functional recovery (Tsai et al., 2006). There was enhanced tissue penetration in scaffolds that retained micro-scale grooves in the range of 30-50 *u*m (Wong et al. 2008). Thus, changes in macro and micro structure may promote axon guidance in spinal cord injury.

Regenerating axons in spinal cord injury must navigate a three dimensional substrate that results from a spinal cord injury. As implied from two dimensional *in vitro* studies, implanted biomaterial scaffolds should likely have organized three dimension structures to enhance axonal guidance *in vivo*. As previously mentioned, *in*

vitro studies indicated that axonal guidance is enhanced from microstructure approximately no larger than the diameter of a cell (Clark et al., 1991; Miller et al., 2002; Ito et al., 1999). However, if the spinal cord lesion cavity must incorporate support cells, the scaffold structure must have pores larger than a cell diameter. Therefore, to promote axon guidance, the scaffold must promote cellular organization or linearity within the channels, lumens, or pores. To promote cellular organization, uniform stresses and strains can be applied upon the cells (Fung, 1997). Therefore, a highly uniform scaffold microstructure may apply uniform stress distributions upon the seeded cells and migratory cells within the channels, promoting an organized cellular microstructure within the lesion to enhance axon guidance. Although many scaffolds have been fabricated with the intent of creating a three dimensional guidance structure (Teng et al., 2002; Patist et al., 2004; Yoshi et al., 2003; Flynn et al., 2003; Tsai et al., 2004), none have been shown to produce consistent microstructures and robust axonal guidance. Thus, there is a theoretical need for a scaffold with physically precise microstructure to promote cellular organization within the lesion, which may consequently enhance axonal guidance and other chemical guidance therapies.

Overview of peripheral nerve injury

There are approximately 20,000 peripheral nerve repair procedures a year in the United States. Peripheral nerve injury has a socio/economic impact as it results in a large number of nonfunctional or disability work days. Furthermore, nearly 3% of the

patients have a life long disability (Noble et al., 1998).

A key difference between the central nervous system and the peripheral nervous system is the innate ability of axons in the peripheral nervous system to regenerate after injury. Axons within the peripheral nerve are capable of regenerating across the lesion site and reforming functional connections, while axons within a spinal cord injury do not readily regenerate through a lesion injury site (Schmidt and Leach, 2003), due to several mechanisms: 1) Regeneration associated genes are more readily upregulated in neurons within a peripheral nerve injury than in the spinal cord (Fernandes and Tetzlaff, 2000). 2) Macrophage removal of myelin debris, which has inhibitory properties, is delayed in the spinal cord than in the peripheral nerve (Schmidt and Leach, 2003). 3) Reactive astrocytes form a disorganized inhibitory scar within the spinal cord lesion (Tator et al., 1991). In contrast, Schwann cells form a guiding structure across peripheral injuries (Oudega et al., 1994). 4) Schwann cells produce growth factors after peripheral injury to attract and guide regenerating axons. Thus, compared to the central nervous system, the peripheral nervous system has an enhanced innate capacity for axonal regeneration across a site of injury.

Peripheral nerve injury is often treated by suturing of the damaged nerve ends or, for lesions greater than 4mm, a nerve autograft (Yu and Bellamkonda, 2003; Lundborg, 1988; Mackinnon and Dellon, 1988). Specifically, suturing nerves ends together beyond a distance of 4mm results in tension which degrades the regenerative capacity of the nerve ends. Thus, a nerve autograft is used in distances beyond 4mm to reduce tension and increase axon regeneration (Millesi, 1976). However, the nerve

autograft results in a second surgery to the patient. To address the scenarios where a nerve autograft is not available, some FDA alternates have been approved, such as the “Nerve Guide” and “SaluBridge Nerve Cuff”. However, these nerve conduits are only used as short distance grafts, and do not replace the superior results from the nerve autograft (Schmidt and Leach, 2003). Thus there is a need for conduits to functionally mimic the nerve autograft over longer distances. Although the nerve autograft is the current “gold standard”, there is a dramatic reduction of functional recovery beyond a 1cm distance, resulting in an unmet need that may be addressed by future conduit designs (Evans, 2001). Therefore, bioengineering strategies aim to develop nerve conduits for long (>1-cm) defects by attempting to mimic the nerve autograft while accelerating axonal growth rates to enhance long distance functional recovery.

Immediately after peripheral nerve injury, chromatolysis occurs, the neuronal cell bodies increase in size and the nucleus migrates to the edge of the cell, enhancing the protein and RNA production. Schwann cells divide and help macrophages remove myelin debris through phagocytosis. Additionally, Schwann cells migrate into remaining connective tissue basement membranes that form endoneurial tubes. The Schwann cells migrate into the endoneurial tubes and form an organized cellular microstructure. These structures are known as the bands of Bunger, and are thought to provide axonal guidance. Additionally, axonal sprouting occurs from the proximal end of the injury at a rate of 2mm/day. Specifically, the sprouts cross the nodes of Ranvier next to intact axons, then enter into the lesion site and beyond. The axons are known to navigate within the basil lamina, along the Schwann cells. After months, the

regenerating nerve is separated into nerve bundles that mimic the innate endoneurial environment. Eventually, some axons reinnervate their target, while other non terminated axons are degraded (Evans, 2001; Fawcett and Keynes, 1990; Lunborg, 1988).

Aspects of peripheral nerve conduit design

Biocompatibility

To replace the nerve autograft, nerve conduits must be made of a biocompatible material. Many natural and synthetic materials have been used to produce a nerve conduit including fibronectin (Whitworth et al., 1995), collagen (Archibald et al., 1991), poly(l-lactic acid) (Evans et al., 2002), and poly phosphoester (Wang et al., 2001). However, no materials have been able to replace the effectiveness of the nerve autograft (Schmidt and Leach, 2003). This may be because natural materials often have batch variability that can produce an immune response and synthetic materials can cause immune responses from degradation products and chemical leaching (Hudson et al., 2000). Additionally, cells or nerve implants from other people or animals can produce a foreign body immune response. Although immunosuppressive drugs have been used to permit implantation of foreign materials, they can be expensive and have variable effectiveness between patients (Berd, 1982). The elasticity of the biomaterial may also affect the biocompatibility, as different mechanical properties along the scaffold/host interface may cause agitation after

movement which can result in an immune response (Ratner, 1997). Furthermore, conduits can degrade under long term storage conditions, rendering them less useful for clinical applications (Gulati et al., 1996). Thus, an ideal biocompatible material would, be produced from a material source with minimal batch variation, solely present inert surface molecules, degrade in a non-reactive manner, reduce mechanical agitation at the host interface, and be capable of long term storage.

Mechanical guidance

Regenerating axons not only need to cross the lesion site, but they need to reinnervate with the appropriate target for functional recovery. However, guiding regenerating axons across a lesion is thought to increase the number of axons reaching their appropriate target. Supporting this, nerve crush injuries, which retain their endoneurial tubes as mechanical guidance, have a greater amount of functional recovery than laceration injuries, with disrupted and misaligned endoneurial tubes (Sunderland, 1978; Haftek and Thomas, 1968). Therefore, when considering a conduit design, it may be important to align the endoneurial tubes across the lesion site to further enhance functional recovery (Alluin et al., 2009).

Although the extent of nerve guidance conduits has been macro level conduit shells, some microstructural properties can be engineered in natural and synthetic materials (Evans, 2002). It is thought that microstructural properties of conduits can affect the ability of axons to proliferate (Doolabh et al., 1996; Guenard et al., 1991; Gao et al., 2005;), and potentially serve as surrogate endoneurial tubes across large

lesions. Specifically, micro fibers and channels have been investigated to enhance axon guidance (Hadlock, 2000; Evans et al., 1999; Sinis et al., 2007). However, these approaches result in inconsistent microenvironment and axonal guidance.

Chemical etching and casting methods can be used to create micron level precision in material design (Hutmacher, 2001). Specifically, biomaterials can be casted into molds to create micron level architecture to promote axon guidance (Stokols et al., 2006). In vitro, axons have been shown to be directed by dimensions similar to a cellular diameter (Miller et al., 2002; Houchin-Ray et al., 2007; Shaw and Shoichet, 2003). Therefore, and ideal biomaterial should likely contain guidance cues that create an environment with linearity no larger than the cellular level. Axons are thought to be guided in part from the cellular organized of the Schwann cells in the nodes of Bunger. Thus, guidance channels which support cells should promote an organized cellular deposition (i.e. Schwann cells) within the conduit to enhance axonal guidance. The external stresses and strains upon a cell can affect its orientation (Fung, 1997). Therefore, to enhance axon guidance, a conduit should be designed to exert uniform stresses and strains upon the cellular environment to promote an organized microenvironment within the lesion site.

Substrate delivery

Many substrates have been delivered within the conduit to promote axon regeneration. To potentially mimic the conditions of a nerve autograft, cells have been seeded within nerve conduits (Evans, 2002). Specifically, Schwann cells are largely

thought to enhance nerve conduits due of their role in forming the nodes of Bunger and release of bioactive molecules. Consequently, there has been an extensive amount of research in implanting Schwann cells within sites of nerve injury (Hadlock et al., 2000; Mosahebi et al., 2002; Evans et al., 2002). Schwann cells potentially enhance axon growth because they produce extracellular matrix molecules (laminin and collagen), cell adhesion molecules (L1, N-cadherin), and neurotrophic factors (NGF, BDNF) thought to guide axon growth (Faweet et al., 1990).

Direct delivery of the extracellular matrix molecules thought to enhance axon growth is an alternative to implanting Schwann cells within the conduit. Specifically, fibronectin (Mosahebi et al., 2003), collagen (Archibald et al., 1991), and laminin (Toba et al., 2002) have been shown to support axon growth within peripheral nerve conduits. Delivery of extracellular matrix molecules provides a more efficient alternative to cellular grafts, reducing the need for cell harvesting and culture while further minimizing the risk of an immune response common to cellular allografts.

Futhermore, fibrin substrate must fill any empty space within the lesion gap or conduit before axons can growth across a peripheral nerve gap (Williams et al., 1983). Therefore, substrate fill into the empty scaffold channels or lumen may reduce the time needed for the fibrin fill, and thus axon growth across the empty lesion gap. This could theoretically enhance functional recovery by increasing the number of axons reaching their distal target before they become nonreceptive to reinnervation. This approach mimics nerve autografts, which innately have a cellular substrate within the

guiding endoneurial tubes. Thus, a conduit design should be capable of supporting a substrate fill within the lumen to further enhance axon growth.

Molecular delivery

Molecules within a peripheral injury are though to enhance regenerating axon growth and guidance. During peripheral nerve injury, Schwann cells release a milieu of neurotrophic factors thought to promote axon growth (Evans, 2002). As previously mentioned, growth factors are thought to guide axons in stages of nerve development (Behar and Barrett, 1997; Tucker et al., 2001; Fritzsch et al., 1997) and have shown promise in guiding extending axons from injured neurons in the spinal cord (Taylor et al., 2006). Additionally, neurotrophins, such as NT-3 (Sterne et al., 1997) and NGF (Xu et al., 2003), have been shown to promote axon growth across peripheral nerve conduits.

To further mimic the functionality of the nerve autograft, a conduit will likely need to supply growth factors to promote axon growth and survival (Evans, 2002). Furthermore, the release of growth factors from the conduit should likely mimic the Schwann cell, which has been heavily associated with axon regeneration (Faweet and Keynes, 1990). The Schwann cell neurotrophin release profiles are likely non-toxic, at concentrations which are chemoattractive (as some molecular signals become repulsive at the wrong concentration), remain bioactive, and are released over an optimal duration to promote axon growth. Thus, a nerve conduit would ideally be

capable of releasing growth factors in a highly controlled, transient manner, through a mechanism that retains molecular bioactivity.

Scaffolds for mechanical guidance of nerve regeneration

Some challenges to nerve regeneration are being addressed through the engineering of biocompatible guidance scaffolds. Specifically, in peripheral nerve regeneration, scaffolds are needed to replace the nerve autograft to remove the need for a second surgery or further enhance functional recovery (Hadlock et al., 1999). Within the central nervous system, scaffolds may provide an organized substrate within the lesion site to enhance axonal guidance across the injury (Schwabb, 2002). Generally, to enhance axon regeneration, scaffolds are engineered to have guidance properties such as; 1) support of cellular substrate, 2) release of growth factors, 4) support of extracellular substrate, 5) axon stimulating electrical properties, or 6) mechanical guidance channels (Schmidt and Leach, 2003). Many scaffolds have been designed to address these approaches and have shown some success during *in vivo* conditions. Below we address scaffolds designed to promote mechanical guidance of axons across a lesion site.

It is commonly accepted that mechanical axon guidance across a lesion site can further promote regeneration (Schmidt and Leach, 2003). This was first shown by Millesi, when he developed a microsurgical method to suture nerve fascicles ends together to maintain the innate guidance channels within the peripheral nerve, thus

enhancing axon regeneration and functional recovery (Millesi, 1976). Later, *in vitro*, Clark showed that axons could detect and be guided by micron level mechanical guidance cues (Clark et al., 1991). Therefore, it is possible that mimicking the innate linearity within the intact spinal cord may further enhance axon regeneration across spinal cord lesions. Recently, scaffolds to guide axons in a linear manner have been designed with internal structures consisting of fibers or open pores (Archbald et al., 1991; Sinis et al., 2007; Hadlock, 2000; Evans et al., 1999). Below we address the ability of scaffolds to enhance linear guidance across a lesion using natural and synthetic materials.

Natural in vivo guidance scaffolds

There are a growing number of natural scaffolds that have made to enhance linear guidance across a lesion site. Generally, natural materials are more biocompatible than synthetic ones, more readily support cellular migration, and have a minimal toxic effect (Evans, 2001). Many scaffolds made of natural extracellular matrix (ECM) components have been used in attempts to promote linear axonal growth and guidance across peripheral nerve and spinal cord lesions. For example, collagen, an ECM connective tissue protein, was shown to enhance axon growth in a spinal cord lesion when formed into organized cylindrical bundles (Yoshi and Oki, 2001). Additionally, collagen fibers were magnetically aligned to enhance linearity within the lesion site and resulted in enhanced axon growth in spinal cord lesions (Ceballos et al., 1999). Furthermore, collagen based NeuraGen and SaluMedia

conduits, have been FDA approved for nerve regeneration in the peripheral nervous system. However these approaches only provide one large guidance channel and are consequently used only for short gaps when an autograft unavailable (Schmidt and Leach, 2003). Scaffolds have also been made from other natural materials sources shown to elicit *in vivo* axon growth. Specifically, chitosan, a structural component of shell fish, was formed into guidance tubes and was shown to support axon regeneration within the rodent peripheral nervous system (Itoh et al., 2003). Although many of these approaches have provided a mechanical guidance substrate to promote axon linearity, none show strict linearity within the lesion site, and often show only gross mechanical guidance. Furthermore, many natural guidance scaffolds may have shown beneficial effects within experimental models, but are not clinically applicable due to the variability in immune response between batches and rapid degradation which results in a short shelf-life (Hudson et al., 2000). Consequently, there are no multi-channel guidance scaffolds approved for use in the peripheral and central nervous system. Thus, there remains a need to further improve naturally based nerve guidance scaffolds to provide linear axonal guidance to enhance regeneration and functional recovery.

Synthetic in vivo guidance scaffolds

To address some of the innate issues of natural guidance scaffolds, synthetic scaffolds have been used to guide axon regeneration *in vivo*. Synthetic materials often provide more control of biodegradation, mechanical strength, and spatial configuration

(Evans, 2001). Specifically, the microgeometry of synthetic microchannels is thought to affect peripheral nerve regeneration (Aebischer et al., 1990). One example is a synthetic biodegradable scaffold with guidance channels composed of poly(lactic-co-glycolic acid) (PLGA), which supported axon growth in spinal cord injury (Moore et al., 2006). A synthetic hydrogel, poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA), was formed into guidance tubes that were shown to enhance axonal regeneration in a spinal cord lesion (Tsai et al., 2004). Additionally, guidance channels made from acrylic copolymer (AC) and silicone elastomer (SE) were shown to support axon guidance within the peripheral nerve injury (Aebischer et al., 1988). Furthermore, Poly(acrylonitile-co-vinylchloride) (PAN/PVC) materials, known to be highly stable and non toxic, have been formed into guidance channels and were shown to enhance axon regeneration in the spinal cord when combined with Schwann cells (Bunge, 1995). Although some of these synthetic guidance materials have been shown to promote axon regeneration, axon regeneration is inconsistent and not strictly linear. Additionally, many of these approaches are either non degradable or their degradation products and manufacturing by-products later leach into the injury, causing an immune response (Hudson et al, 2000). Currently there are no clinically used synthetic guidance scaffolds for peripheral or central nervous system repair, thus there remains a need to further develop synthetic materials to further enhance axon regeneration and functional recovery.

Although many biomaterials have been tested *in vivo* to promote linear axons guidance, none have been able to address the current challenges in nerve regeneration;

1) To replace the nerve autograft for peripheral injury or 2) Elicit functional recovery in human spinal cord injuries. Future development of natural or synthetic guidance scaffolds should aim to be biocompatible and enhance axon linearity across lesion site to to mimic the innate linearity within the intact spinal cord, while potentially being capable of biodegradability, cellular substrates, non cellular substrates, appropriate mechanical stiffness, and growth factor delivery to further promote axonal guidance.

Rationale and hypotheses

Despite the complex interaction of molecular and physical guidance cues in vivo, studies demonstrating 1) the growth stimulating effect of neurotrophins on injured axons in the spinal cord and peripheral nerves, 2) the effectiveness of physical guidance cues obtained from physical substrates on neurite extension in vitro, lead to the following hypothesis: **nerve guidance scaffolds with a precise physical architecture can be used to guide axons across a lesion site within the spinal cord and peripheral nerve, and if coupled with neurotrophic proteins, can stimulate robust, long tract, axonal growth**. Thus, the overall goal of this thesis was to combine our linearized agarose scaffold with neurotrophin and gene upregulation therapies, to *stimulate and guide* axonal regeneration across and *beyond* the lesion site following spinal cord injury (SCI) and peripheral nerve injury (PNI). To achieve this goal, the following specific aims were addressed:

Specific Aim 1: Test a templated agarose nerve guidance scaffold with a combinatorial therapy of a neurotrophin gradient (NT-3) and conditioning lesion in an *in vivo* model of SCI for their ability to stimulate and guide long-tract, *ascending* axon regeneration: The scaffolds with a combinatorial therapy of a conditioning lesion and a neurotrophin gradient containing NT-3 will be tested *in vivo* for their ability to stimulate and guide robust long-tract ascending axon regeneration in an *in vivo* model of SCI. Fischer 344 female rats will be subjected to dorsal column lesions of the spinal cord. Scaffolds will be implanted, the combinatorial treatment applied, and after surviving for one month, animals will be perfused with 4% PFA and spinal cord tissue will be prepared for histology. The overall tissue morphology, inflammation, cellular infiltration, vascularization, and extent to which axonal regeneration is linear will be evaluated.

Specific Aim 2: Test a templated nerve guidance scaffold with a combinatorial therapy of a neurotrophin gradient (BDNF) in an *in vivo* model of SCI for their ability to stimulate and guide, *descending* axon regeneration: The linearized scaffold will be scaled to fit a complete transection. An *in vivo* model for a complete transection spinal cored injury will be used to test the ability of the linearized scaffolds and a neurotrophin gradient containing BDNF for their ability to robustly stimulate and guide descending axon regeneration. Fischer 344 female rats will receive a complete transection lesion at the thoracic-T10 level of the spinal cord. One month after scaffold implantation and gene therapy beyond the lesion site, animals will be

perfused with 4% PFA and spinal cord tissue will be prepared for histology. Again, the overall tissue morphology, inflammation, cellular infiltration, vascularization, and regenerating axon linearity will be evaluated.

Specific Aim 3: Test a nerve conduit for biocompatibility and axon permissiveness within the peripheral nervous system: The previously developed scaffold will be scaled to the length and size of a peripheral nerve and placed into a conduit sleeve before implantation. The conduit will be tested for biocompatibility, stability, and ability to guide axon regeneration in an *in vivo* model of PNI. Fischer 344 female rats will be subjected to a sciatic nerve lesion. Conduits will be implanted and after surviving for one, three, six, and twelve months, animals will be perfused with 4% PFA and peripheral nerve tissue will be prepared for histology. Similarly to the above aims, the tissue morphology, cellular infiltration, vascularization, axon penetration, and inflammation will be evaluated.

Acknowledgement

This work was made possible by grants from the NIH. I would like to thank Dr. Jeff Sakamoto, Dr. Leif Havton, Dr. Armin Blesch, and Dr. Mark Tuszynski for work on this project.

References

Alluin, O, C Wittmann, T Marqueste, J Chabas, S Garcia, M Lavaut, D Guinard, F Feron and P Decherchi (2009) Functional recovery after peripheral nerve injury and implantation of a collagen guide. Biomaterials 30(3): 363-73.

Apostolova, I, A Irintchev and M Schachner (2006) Tenascin-r restricts posttraumatic remodeling of motoneuron innervation and functional recovery after spinal cord injury in adult mice. Journal of Neuroscience 26(30): 7849.

Archibald, S, C Krarup, J Shefner, S Li and R Madison (1991) A collagen-based nerve guide conduit for peripheral nerve repair: An electrophysiological study of nerve regeneration in rodents and nonhuman primates. The Journal of Comparative Neurology 306(4): 685-96.

Barritt, A, M Davies, F Marchand, R Hartley, J Grist, P Yip, S Mcmahon and E Bradbury (2006) Chondroitinase abc promotes sprouting of intact and injured spinal systems after spinal cord injury. Journal of Neuroscience 26(42): 10856.

Becker, T, R Bernhardt, E Reinhard, M Wullimann, E Tongiorgi and M Schachner (1998) Readiness of zebrafish brain neurons to regenerate a spinal axon correlates with differential expression of specific cell recognition molecules. Journal of Neuroscience 18(15): 5789-803.

Behar, T, M Dugich-Djordjevic, Y Li, W Ma, R Somogyi, X Wen, E Brown, C Scott, R Mckay and J Barker (1997) Neurotrophins stimulate chemotaxis of embryonic cortical neurons. European Journal of Neuroscience 9(12): 2561-70.

Berd, D, M Mastrangelo, P Engstrom, A Paul and H Maguire (1982) Augmentation of the human immune response by cyclophosphamide. Cancer Research 42(11): 4862.

Blesch, A, P Lu and M Tuszynski (2002) Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. Brain Research Bulletin 57(6): 833-38.

Bomze, H, K Bulsara, B Iskandar, P Caroni and J Skene (2001) Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nature Neuroscience 4: 38-43.

Bracken, M (2003) Steroids for acute spinal cord injury. Ann Emerg Med 41(3): 410-3.

Bradbury, E, S Khemani, R Von, J Priestley and S Mcmahon (1999) Nt-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. European Journal of Neuroscience 11(11): 3873-83.

Bradbury, E, L Moon, R Popat, V King, G Bennett, P Patel, J Fawcett and S Mcmahon (2002) Chondroitinase abc promotes axon regeneration and functional recovery following spinal cord injury. Nature 416: 636-40.

Broude, E, M Mcatee, M Kelley and B Bregman (1997) C-jun expression in adult rat dorsal root ganglion neurons: Differential response after central or peripheral axotomy. Experimental Neurology 148(1): 367-77.

Buffo, A, A Holtmaat, T Savio, J Verbeek, J Oberdick, A Oestreicher, W Gispen, J Verhaagen, F Rossi and P Strata (1997) Targeted overexpression of the neurite growth-associated protein b-50/gap-43 in cerebellar purkinje cells induces sprouting after axotomy but not axon regeneration into growth-permissive transplants. Journal of Neuroscience 17(22): 8778-91.

Bundesen, L, T Scheel, B Bregman and L Kromer (2003) Ephrin-b2 and ephb2 regulation of astrocyte-meningeal fibroblast interactions in response to spinal cord lesions in adult rats. Journal of Neuroscience 23(21): 7789-800.

Bush, T, N Puvanachandra, C Homer, A Polito, T Ostenfeld, C Svendsen, L Mucke, M Johnson and M Sofroniew (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. NEURON-CAMBRIDGE MA- 23: 297-308.

Cajal, S (1991) Cajal's degeneration and regeneration of the nervous system. History of neuroscience.

Campbell, G, P Anderson, M Turmaine and A Lieberman (1991) Gap-43 in the axons of mammalian cns neurons regenerating into peripheral nerve grafts. Experimental Brain Research 87(1): 67-74.

Ceballos, D, X Navarro, N Dubey, G Wendelschafer-Crabb, W Kennedy and R Tranquillo (1999) Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration. Experimental Neurology 158(2): 290-300.

Chen, Z, M Negra, A Levine, Y Ughrin and J Levine (2002) Oligodendrocyte precursor cells: Reactive cells that inhibit axon growth and regeneration. Journal of Neurocytology 31(6): 481-95.

Chopp, M, X Zhang, Y Li, L Wang, J Chen, D Lu, M Lu and M Rosenblum (2000) Spinal cord injury in rat: Treatment with bone marrow stromal cell transplantation. NeuroReport 11(13): 3001.

Clark, P (1991) Cell guidance by ultrafine topography in vitro. Journal of Cell Science 99(1): 73-77.

Clark, P (1992) Cell guidance by micropatterned adhesiveness in vitro. Journal of Cell Science 103(1): 287-92.

Clark, P (1993) Growth cone guidance and neuron morphology on micropatterned laminin surfaces. Journal of Cell Science 105(1): 203-12.

Cybulski, G, J Stone and R Kant (1989) Outcome of laminectomy for civilian gunshot injuries of the terminal spinal cord and cauda equina: Review of 88 cases. Neurosurgery 24(3): 392.

Doolabh, V, M Hertl and S Mackinnon (1996) The role of conduits in nerve repair: A review. Rev Neurosci 7(1): 47-84.

Dou, C and J Levine (1994) Inhibition of neurite growth by the ng2 chondroitin sulfate proteoglycan. Journal of Neuroscience 14(12): 7616-28.

Dumont, R, D Okonkwo, S Verma, R Hurlbert, P Boulos, D Ellegala and A Dumont (2001) Acute spinal cord injury, part i: Pathophysiologic mechanisms. Clinical Neuropharmacology 24(5): 254.

Ellis-Behnke, R, Y Liang, S You, D Tay, S Zhang, K So and G Schneider (2006) Nano neuro knitting: Peptide nanofiber scaffold for brain repair and axon regeneration with functional return of vision. Proceedings of the National Academy of Sciences 103(13): 5054-59.

Evans, G (2001) Peripheral nerve injury: A review and approach to tissue engineered constructs. The Anatomical Record 263(4): 396-404.

Evans, G, K Brandt, S Katz, P Chauvin, L Otto, M Bogle, B Wang, R Meszlenyi, L Lu and A Mikos (2002) Bioactive poly (l-lactic acid) conduits seeded with schwann cells for peripheral nerve regeneration. Biomaterials 23(3): 841-48.

Evans, G, K Brandt, M Widmer, L Lu, R Meszlenyi, P Gupta, A Mikos, J Hodges, J Williams and A Gürlek (1999) In vivo evaluation of poly (l-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 20(12): 1109-15.

Faweett, J and R Keynes (1990) Peripheral nerve regeneration. Annual Reviews in Neuroscience 13(1): 43-60.

Fehlings, M, L Sekhon and C Tator (2001) The role and timing of decompression in acute spinal cord injury: What do we know? What should we do? Spine 26(24S): S101.

Fehlings, M and C Tator (1999) An evidence-based review of decompressive surgery in acute spinal cord injury: Rationale, indications, and timing based on experimental and clinical studies. J Neurosurg Spine 91(1): 1–11.

Fernandes, K and W Tetzlaff (2000) Gene expression in axotomized neurons: Identifying the intrinsic determinants of axonal growth. Axonal Regeneration in the Central Nervous System: 219–66.

Fitch, M and J Silver (1997) Glial cell extracellular matrix: Boundaries for axon growth in development and regeneration. Cell and Tissue Research 290(2): 379-84.

Flynn, L, P Dalton and M Shoichet (2003) Fiber templating of poly (2-hydroxyethyl methacrylate) for neural tissue engineering. Biomaterials 24(23): 4265-72.

Franklin, R, J Gilson, I Franceschini and S Barnett3s4 (1996) Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult cns. Glia 17: 217-24.

Frey, D, T Laux, L Xu, C Schneider and P Caroni (2000) Shared and unique roles of cap23 and gap43 in actin regulation, neurite outgrowth, and anatomical plasticity. The Journal of Cell Biology 149(7): 1443-54.

Friedman, J, A Windebank, M Moore, R Spinner, B Currier and M Yaszemski (2002) Biodegradable polymer grafts for surgical repair of the injured spinal cord. Neurosurgery 51(3): 742.

Fritzsch, B, I Silos-Santiago, L Bianchi and I Fariñas (1997) The role of neurotrophic factors in regulating the development of inner ear innervation. Trends in Neurosciences 20(4): 159-64.

Fung, Y and Asomeam Division. (1997). Biomechanics, Springer New York.

Gallo, G, F Lefcort and P Letourneau (1997) The trka receptor mediates growth cone turning toward a localized source of nerve growth factor. Journal of Neuroscience 17(14): 5445-54.

Gao, S, S Wang and S Ramakrishna (2005) Development of fibrous biodegradable polymer conduits for guided nerve regeneration. Journal of Materials Science: Materials in Medicine 16(4): 367-75.

Gerndt, S, J Rodriguez, J Pawlik, P Taheri, W Wahl, A Micheals and S Papadopoulos (1997) Consequences of high-dose steroid therapy for acute spinal cord injury. The Journal of Trauma: Injury, Infection, and Critical Care 42(2): 279.

Giehl, K, A Schutte, P Mestres and Q Yan (1998) The survival-promoting effect of glial cell line-derived neurotrophic factor on axotomized corticospinal neurons in vivo is mediated by an endogenous brain-derived neurotrophic factor mechanism. Journal of Neuroscience 18(18): 7351-60.

Giehl, K and W Tetzlaff (1996) Bdnf and nt-3, but not ngf, prevent axotomy-induced death of rat corticospinal neurons in vivo. European Journal of Neuroscience 8(6): 1167-75.

Goodman, C (1996) Mechanisms and molecules that control growth cone guidance. Annual Reviews in Neuroscience 19(1): 341-77.

Grimpe, B and J Silver (2004) A novel DNA enzyme reduces glycosaminoglycan chains in the glial scar and allows microtransplanted dorsal root ganglia axons to regenerate beyond lesions in the spinal cord. Journal of Neuroscience 24(6): 1393-97.

Guenard, V, R Valentini and P Aebischer (1991) Influence of surface texture of polymeric sheets on peripheral nerve regeneration in a two-compartment guidance system. Biomaterials 12(2): 259-63.

Gulati, A (1996) Peripheral nerve regeneration through short-and long-term degenerated nerve transplants. Brain Research 742(1-2): 265-70.

Gundersen, R and J Barrett (1979) Neuronal chemotaxis: Chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206(4422): 1079-80.

Hadlock, T, J Elisseeff, R Langer, J Vacanti and M Cheney (1998) A tissue-engineered conduit for peripheral nerve repair. Archives of Otolaryngology-Head and Neck Surgery 124(10): 1081-86.

Hadlock, T, C Sundback, D Hunter, M Cheney and J Vacanti (2000) A polymer foam conduit seeded with schwann cells promotes guided peripheral nerve regeneration. Tissue Engineering 6(2): 119-27.

Houchin-Ray, T, K Whittlesey and L Shea (2007) Spatially patterned gene delivery for localized neuron survival and neurite extension. Molecular Therapy 15(4): 705.

Houle, J, V Tom, D Mayes, G Wagoner, N Phillips and J Silver (2006) Combining an autologous peripheral nervous system" bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. Journal of Neuroscience 26(28): 7405.

Hudson, T, G Evans and C Schmidt (2000) Engineering strategies for peripheral nerve repair. Orthopedic Clinics of North America 31(3): 485-97.

Hurlbert, R (2001) The role of steroids in acute spinal cord injury: An evidence-based analysis. Spine 26(24S): S39.

Hutmacher, D (2001) Scaffold design and fabrication technologies for engineering tissues—state of the art and future perspectives. Journal of Biomaterials Science, Polymer Edition 12(1): 107-24.

Iannotti, C, H Li, P Yan, X Lu, L Wirthlin and X Xu (2003) Glial cell line-derived neurotrophic factor-enriched bridging transplants promote propriospinal axonal regeneration and enhance myelination after spinal cord injury. Experimental Neurology 183(2): 379-93.

Im Ozgenel, G (2003) Effects of hyaluronic acid on peripheral nerve scarring and regeneration in rats. Microsurgery 23: 575-81.

Ito, Y, T Yagi, H Kanda, S Tanaka, M Watanabe and Y Uchikawa (1999). Cultures of neurons on micro-electrode array in hybrid retinalimplant. Systems, Man, and Cybernetics, 1999. IEEE SMC'99 Conference Proceedings. 1999 IEEE International Conference on.

Itoh, S, M Suzuki, I Yamaguchi, K Takakuda, H Kobayashi, K Shinomiya and J Tanaka (2003) Development of a nerve scaffold using a tendon chitosan tube. Artificial Organs 27(12): 1079-88.

Jin, Y, I Fischer, A Tessler and J Houle (2002) Transplants of fibroblasts genetically modified to express bdnf promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Experimental Neurology 177(1): 265-75.

Jones, L, R Margolis and M Tuszynski (2003) The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Experimental Neurology 182(2): 399-411.

Jones, L, M Oudega, M Bunge and M Tuszynski (2001) Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. The Journal of Physiology 533(1): 83-89.

Korsching, S (1993) The neurotrophic factor concept: A reexamination. Journal of Neuroscience 13(7): 2739-48.

Leach, J and C Schmidt (2005) Characterization of protein release from photocrosslinkable hyaluronic acid-polyethylene glycol hydrogel tissue engineering scaffolds. Biomaterials 26(2): 125-35.

Leon, S, Y Yin, J Nguyen, N Irwin and L Benowitz (2000) Lens injury stimulates axon regeneration in the mature rat optic nerve. Journal of Neuroscience 20(12): 4615.

Levine, J, R Reynolds and J Fawcett (2001) The oligodendrocyte precursor cell in health and disease. Trends in Neurosciences 24(1): 39-47.

Lindvall, O, Z Kokaia and A Martinez-Serrano (2004) Stem cell therapy for human neurodegenerative disorders–how to make it work. NEURODEGENERATION: S43.

Lu, J, F Feron, A Mackay-Sim and P Waite (2002) Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. Brain 125(1): 14.

Lu, P, L Jones and M Tuszynski (2005) Bdnf-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Experimental Neurology 191(2): 344-60.

Lu, P, H Yang, L Jones, M Filbin and M Tuszynski (2004) Combinatorial therapy with neurotrophins and camp promotes axonal regeneration beyond sites of spinal cord injury. Journal of Neuroscience 24(28): 6402-09.

Lundborg, G (1988). Nerve injury and repair, Churchill Livingstone New York.

Lundborg, G (2000) A 25-year perspective of peripheral nerve surgery: Evolving neuroscientific concepts and clinical significance. Journal of Hand Surgery 25(3): 391-414.

Lundborg, G, L Dahlin, N Danielsen, R Gelberman, F Longo, H Powell and S Varon (1982) Nerve regeneration in silicone chambers: Influence of gap length and of distal stump components. Exp Neurol 76(2): 361-75.

Mackinnon, S and A Dellon (1988). Surgery of the peripheral nerve, Thieme Medical Publishers.

Madsen, J, P Macdonald, N Irwin, D Goldberg, G Yao, K Meiri, I Rimm, P Stieg and L Benowitz (1998) Tacrolimus (fk506) increases neuronal expression of gap-43 and improves functional recovery after spinal cord injury in rats. Experimental Neurology 154(2): 673-83.

Maquet, V, D Martin, B Malgrange, R Franzen, J Schoenen, G Moonen and R Jerome (2000) Peripheral nerve regeneration using bioresorbable macroporous polylactide scaffolds. Journal of Biomedical Materials Research 52(4): 639-51.

Massey, J, C Hubscher, M Wagoner, J Decker, J Amps, J Silver and S Onifer (2006) Chondroitinase abc digestion of the perineuronal net promotes functional collateral sprouting in the cuneate nucleus after cervical spinal cord injury. Journal of Neuroscience 26(16): 4406-14.

Mcdonald, J and C Sadowsky (2002) Spinal-cord injury. The Lancet 359(9304): 417-25.

Mckeon, R, M Jurynec and C Buck (1999) The chondroitin sulfate proteoglycans neurocan and phosphacan are expressed by reactive astrocytes in the chronic cns glial scar. Journal of Neuroscience 19(24): 10778.

Meiri, K and D Burdick (1991) Nerve growth factor stimulation of gap-43 phosphorylation in intact isolated growth cones. Journal of Neuroscience 11(10): 3155-64.

Meiri, K, J Saffell, F Walsh and P Doherty (1998) Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (gap-43) function and is associated with gap-43 phosphorylation in growth cones. Journal of Neuroscience 18(24): 10429-37.

Menei, P, C Montero-Menei, S Whittemore, R Bunge and M Bunge (1998) Schwann cells genetically modified to secrete human bdnf promote enhanced axonal regrowth across transected adult rat spinal cord. European Journal of Neuroscience 10(2): 607-21.

Miller, C, S Jeftinija and S Mallapragada (2002) Synergistic effects of physical and chemical guidance cues on neurite alignment and outgrowth on biodegradable polymer substrates. Tissue Engineering 8(3): 367-78.

Millesi, H (1976) Further experience with interfascicular grafting of the median, ulnar, and radial nerves. The Journal of Bone and Joint Surgery 58(2): 209-18.

Millesi, H (1990) Progress in peripheral nerve reconstruction. World Journal of Surgery 14(6): 733-47.

Moore, M, J Friedman, E Lewellyn, S Mantila, A Krych, S Ameenuddin, A Knight, L Lu, B Currier and R Spinner (2006) Multiple-channel scaffolds to promote spinal cord axon regeneration. Biomaterials 27(3): 419-29.

Mosahebi, A, P Fuller, M Wiberg and G Terenghi (2002) Effect of allogeneic schwann cell transplantation on peripheral nerve regeneration. Experimental Neurology 173(2): 213-23.

Mosahebi, A, M Wiberg and G Terenghi (2003) Addition of fibronectin to alginate matrix improves peripheral nerve regeneration in tissue-engineered conduits. Tissue Engineering 9(2): 209-18.

Nakahara, Y, F Gage and M Tuszynski (1996) Grafts of fibroblasts genetically modified to secrete ngf, bdnf, nt-3, or basic fgf elicit differential responses in the adult spinal cord. Cell Transplant 5(2): 191-204.

Ness, R and S David (1997) Leptomeningeal cells modulate the neurite growth promoting properties of astrocytes in vitro. Glia 19(1): 47-57.

Neumann, S, F Bradke, M Tessier-Lavigne and A Basbaum (2002) Regeneration of sensory axons within the injured spinal cord induced by intraganglionic camp elevation. Neuron 34(6): 885-93.

Neumann, S and C Woolf (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23(1): 83-91.

Niclou, S, E Franssen, E Ehlert, M Taniguchi and J Verhaagen (2003) Meningeal cell-derived semaphorin 3a inhibits neurite outgrowth. Molecular and Cellular Neuroscience 24(4): 902-12.

Noble, J, C Munro, V Prasad and R Midha (1998) Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. The Journal of Trauma: Injury, Infection, and Critical Care 45(1): 116.

Nomura, H, C Tator and M Shoichet (2006) Bioengineered strategies for spinal cord repair. Journal of Neurotrauma 23(3-4): 496-507.

Oudega, M, S Varon and T Hagg (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: Promoting effects of conditioning lesion and graft predegeneration. Exp Neurol 129(2): 194-206.

Pasterkamp, R, P Anderson and J Verhaagen (2001) Peripheral nerve injury fails to induce growth of lesioned ascending dorsal column axons into spinal cord scar tissue expressing the axon repellent semaphorin3a. European Journal of Neuroscience 13(3): 457-71.

Pasterkamp, R, R Giger, M Ruitenberg, A Holtmaat, J De Wit, F De Winter and J Verhaagen (1999) Expression of the gene encoding the chemorepellent semaphorin iii is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal cns. Molecular and Cellular Neuroscience 13(2): 143-66.

Patist, C, M Mulder, S Gautier, V Maquet, R Jérôme and M Oudega (2004) Freeze-dried poly (d, l-lactic acid) macroporous guidance scaffolds impregnated with brain-derived neurotrophic factor in the transected adult rat thoracic spinal cord. Biomaterials 25(9): 1569-82.

Pizzi, M and M Crowe (2006) Transplantation of fibroblasts that overexpress matrix metalloproteinase-3 into the site of spinal cord injury in rats. Journal of Neurotrauma 23(12): 1750-65.

Plunet, W, B Kwon and W Tetzlaff (2002) Mini-review promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. Journal of Neuroscience Research 68: 1-6.

Qiu, J, D Cai, H Dai, M Mcatee, P Hoffman, B Bregman and M Filbin (2002) Spinal axon regeneration induced by elevation of cyclic amp. Neuron 34(6): 895-903.

Quencer, R and R Bunge (1996) The injured spinal cord: Imaging, histopathologic, clinical correlates, and basic science approaches to enhancing neural function after spinal cord injury. Spine 21(18): 2064.

Ramon, Y and S Cajal (1928) Degeneration and regeneration of the nervous system. Translation of.

Ratner, B (1997). Biomaterials science: An introduction to materials in medicine, Academic Press.

Rhodes, K and J Fawcett (2004) Chondroitin sulphate proteoglycans: Preventing plasticity or protecting the cns? Journal of Anatomy 204(1): 33-48.

Richardson, P and V Issa (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309(5971): 791-93.

Richardson, P, U Mcguinness and A Aguayo (1980) Axons from cns neurones regenerate into pns grafts. Nature 284(5753): 264-65.

Rochkind, S, A Shahar, M Amon and Z Nevo (2002) Transplantation of embryonal spinal cord nerve cells cultured on biodegradable microcarriers followed by low power laser irradiation for the treatment of traumatic paraplegia in rats. Neurol Res 24(4): 355-60.

Ronsyn, M, Z Berneman, V Van Tendeloo, P Jorens and P Ponsaerts (2008) Can cell therapy heal a spinal cord injury? Spinal Cord.

Schmidt, C and J Leach (2003) N eural t issue e ngineering: Strategies for repair and regeneration. Annual Reviews in Biomedical Engineering 5(1): 293-347.

Schnell, L, R Schneider, R Kolbeck, Y Barde and M Schwab (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367(6459): 170-73.

Schreyer, D and J Skene (1993) Injury-associated induction of gap-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. Journal of neurobiology 24(7): 959-70.

Schwab, M (2002) Repairing the injured spinal cord. Science 295(5557): 1029-31.

Shaw, D and M Shoichet (2003) Toward spinal cord injury repair strategies: Peptide surface modification of expanded poly (tetrafluoroethylene) fibers for guided neurite outgrowth in vitro. Journal of Craniofacial Surgery 14(3): 308.

Shearer, M and J Fawcett (2001) The astrocyte/meningeal cell interface-a barrier to successful nerve regeneration? Cell and Tissue Research 305(2): 267-73.

Shibayama, M, S Hattori, B Himes, M Murray and A Tessler (1998) Neurotrophin-3 prevents death of axotomized clarke's nucleus neurons in adult rat. The Journal of Comparative Neurology 390: 102-11.

Short, D, W El Masry and P Jones (2000) High dose methylprednisolone in the management of acute spinal cord injury-a systematic review from a clinical perspective. Spinal Cord 38(5): 273-86.

Silver, J (1994) Inhibitory molecules in development and regeneration. Journal of Neurology 242: 22-24.

Sinis, N, O Guntinas-Lichius, A Irintchev, E Skouras, S Kuerten, S Pavlov, H Schaller, S Dunlop and D Angelov (2008) Manual stimulation of forearm muscles does not improve recovery of motor function after injury to a mixed peripheral nerve. Experimental Brain Research 185(3): 469-83.

Sinis, N, H Schaller, C Schulte-Eversum, T Lanaras, B Schlosshauer, M Doser, K Dietz, H Rosner, H Muller and M Haerle (2007) Comparative neuro tissue engineering using different nerve guide implants. Acta Neurochirurgica-Supplementum Then Suppplement-Wein 100: 61.

Smith-Thomas, L (1995) Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors. Journal of Cell Science 108(3): 1307-15.

Song, H and M Poo (1999) Signal transduction underlying growth cone guidance by diffusible factors. Current Opinion in Neurobiology 9(3): 355-63.

Steinmetz, M, K Horn, V Tom, J Miller, S Busch, D Nair, D Silver and J Silver (2005) Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. Journal of Neuroscience 25(35): 8066-76.

Sterne, G, R Brown, C Green and G Terenghi (1997) Neurotrophin-3 delivered locally via fibronectin mats enhances peripheral nerve regeneration. European Journal of Neuroscience 9(7): 1388-96.

Stokols, S and M Tuszynski (2004) The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. Biomaterials 25(27): 5839-46.

Suematsu, N, Y Atsuta and T Hirayama (1988) Vein graft for repair of peripheral nerve gap. J Reconstr Microsurg 4(4): 313-8.

Takami, T, M Oudega, M Bates, P Wood, N Kleitman and M Bunge (2002) Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. Journal of Neuroscience 22(15): 6670.

Tator, C (1995) Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathology 5(4): 407-13.

Taylor, L, L Jones, M Tuszynski and A Blesch (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. Journal of Neuroscience 26(38): 9713.

Taylor, S, J Mcdonald and S Sakiyama-Elbert (2004) Controlled release of neurotrophin-3 from fibrin gels for spinal cord injury. Journal of Controlled Release 98(2): 281-94.

Teng, Y, E Lavik, X Qu, K Park, J Ourednik, D Zurakowski, R Langer and E Snyder (2002) Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proceedings of the National Academy of Sciences 99(5): 3024.

Toba, T, T Nakamura, A Lynn, K Matsumoto, S Fukuda, M Yoshitani, Y Hori and Y Shimizu (2002) Evaluation of peripheral nerve regeneration across an 80-mm gap using a polyglycolic acid(pga): Collagen nerve conduit filled with laminin-soaked collagen sponge in dogs. International journal of artificial organs 25(3): 230-37.

Tobias, C, S Han, J Shumsky, D Kim, M Tumolo, N Dhoot, M Wheatley, I Fischer, A Tessler and M Murray (2005) Alginate encapsulated bdnf-producing fibroblast grafts permit recovery of function after spinal cord injury in the absence of immune suppression. Journal of Neurotrauma 22(1): 138-56.

Tom, V, M Steinmetz, J Miller, C Doller and J Silver (2004) Studies on the development and behavior of the dystrophic growth cone, the hallmark of regeneration failure, in an in vitro model of the glial scar and after spinal cord injury. Journal of Neuroscience 24(29): 6531-39.

Tsai, E, P Dalton, M Shoichet and C Tator (2004) Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection. Journal of Neurotrauma 21(6): 789-804.

Tsai, E, P Dalton, M Shoichet and C Tator (2006) Matrix inclusion within synthetic hydrogel guidance channels improves specific supraspinal and local axonal regeneration after complete spinal cord transection. Biomaterials 27(3): 519-33.

Tucker, K, M Meyer and Y Barde (2001) Neurotrophins are required for nerve growth during development. Nature Neuroscience 4: 29-37.

Tuszynski, M, J Kordower and So Service (1999). Cns regeneration: Basic science and clinical advances, Academic Press.

Vaudano, E, G Campbell, P Anderson, A Davies, C Woolhead, D Schreyer and A Lieberman (1995) The effects of a lesion or a peripheral nerve graft on gap-43 upregulation in the adult rat brain: An in situ hybridization and immunocytochemical study. Journal of Neuroscience 15(5): 3594-611.

Vroemen, M, L Aigner, J Winkler and N Weidner (2003) Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. European Journal of Neuroscience 18(4): 743-51.

Wang, J, M Chuah, D Yew, P Leung and D Tsang (1995) Effects of astrocyte implantation into the hemisected adult rat spinal cord. Neuroscience 65(4): 973-81.

Wang, S, A Wan, X Xu, S Gao, H Mao, K Leong and H Yu (2001) A new nerve guide conduit material composed of a biodegradable poly (phosphoester). Biomaterials 22(10): 1157-69.

Whitworth, I, R Brown, C Doré, C Green and G Terenghi (1995) Orientated mats of fibronectin as a conduit material for use in peripheral nerve repair. Journal of Hand Surgery 20(4): 429-36.

Williams, L, F Longo, H Powell, G Lundborg and S Varon (1983) Spatial-temporal progress of peripheral nerve regeneration within a silicone chamber: Parameters for a bioassay. J Comp Neurol 218(4): 460-70.

Woerly, S, E Pinet, L De Robertis, D Van Diep and M Bousmina (2001) Spinal cord repair with phpma hydrogel containing rgd peptides (neurogel™). Biomaterials 22(10): 1095-111.

Wong, D, J Leveque, H Brumblay, P Krebsbach, S Hollister and F Lamarca (2008) Macro-architectures in spinal cord scaffold implants influence regeneration. Journal of Neurotrauma 25(8): 1027-37.

Xu, X, W Yee, P Hwang, H Yu, A Wan, S Gao, K Boon, H Mao, K Leong and S Wang (2003) Peripheral nerve regeneration with sustained release of poly (phosphoester) microencapsulated nerve growth factor within nerve guide conduits. Biomaterials 24(13): 2405-12.

Y Cajal, S, L Azoulay, Csdi Científicas and Iry Cajal (1911). Histologie du système nerveux de l'homme & des vertébrés, A. Maloine.

Yan, Y, X Wang, Z Xiong, H Liu, F Liu, F Lin, R Wu, R Zhang and Q Lu (2005) Direct construction of a three-dimensional structure with cells and hydrogel. Journal of Bioactive and Compatible Polymers 20(3): 259.

Yick, L, W Wu, K So, H Yip and D Shum (2000) Chondroitinase abc promotes axonal regeneration of clarke's neurons after spinal cord injury. NeuroReport 11(5): 1063.

Yoshii, S and M Oka (2001) Collagen filaments as a scaffold for nerve regeneration. Journal of Biomedical Materials Research 56(3): 400-05.

Yoshii, S, M Oka, M Shima, M Akagi and A Taniguchi (2003) Bridging a spinal cord defect using collagen filament. Spine 28(20): 2346.

Yu, X and R Bellamkonda (2003) Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Engineering 9(3): 421-30.

Yu, X, G Dillon and R Bellamkonda (1999) A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. Tissue Engineering 5(4): 291-304.

Zhang, Y, J Winterbottom, M Schachner, A Lieberman and P Anderson (1997) Tenascin-c expression and axonal sprouting following injury to the spinal dorsal columns in the adult rat. Journal of Neuroscience Research 49(4): 433-50.

CHAPTER 2

Templated agarose scaffolds orient and guide regenerating long-tract axons through sites of spinal cord injury

Abstract

Previously we reported that micron-scale templated agarose scaffolds can orient and guide local spinal cord axons after injury. In the present study we examined whether growth of long-projecting spinal cord axons could also be promoted into, and then beyond, templated agarose scaffolds placed into a spinal cord lesion site. Ascending spinal cord dorsal column sensory axons were transected at the C4 level. Animals were then subjected to combinatorial therapies consisting of: 1) templated agarose scaffolds implanted into the lesion site, seeded with autologous bone marrow stromal cells expressing a growth factor, neurotrophin-3 (NT-3), 2) lentiviral vectors expressing NT-3 beyond the lesion site (to promote axonal emergence from the scaffold along chemotropic gradients of growth factors), and 3) priming lesions ("conditioning lesions") of the sensory neuronal cell body to stimulate the endogenous growth state of the injured neuron. Control groups received either non-organized, NT-3-expressing cell suspension grafts in the lesion site, or templated scaffolds plus one of the two components of the combination therapy. Among groups that received templated agarose scaffolds, long-tract sensory axonal regeneration occurred into the spinal cord lesion site, and the growth of these axons was remarkably organized and

linear compared to non-organized cell suspension grafts. Axonal penetration was maximal in subjects that received combination therapies; further, 83 $\pm$ 13% of axons entering the scaffolds in combination-treated subjects continued to grow the full length of the lesion cavity to reach the distal aspect of the scaffold, over a 2mm distance. In contrast, axons regenerating into cell suspension grafts lacking guidance scaffolds exhibited a parabolic decay of growth as a function of distance, and only 22 $\pm$ 6% of axons extended the length of the lesion cavity. Moreover, axonal regeneration beyond the lesion site occurred only among subjects that received full combinatorial treatments, an effect that was highly significant ($p<0.05$). However, axon growth beyond the scaffold was constrained to a reactive cell layer that formed between the distal aspect of the scaffold and host tissue, and did not continue further to re-penetrate the host spinal cord. Thus, templated agarose scaffolds substantially enhance the organization and distance over which long-tract axons extend through a spinal cord lesion site in the presence of combinatorial therapies, but host-scaffold reactive interfaces limit axon re-penetration of the host. Further development must reduce reactive cellular interfaces to support effective axonal penetration of host parenchyma.

Introduction

The adult mammalian central nervous system (CNS) exhibits little or no capacity to spontaneously regenerate (Ramon, 1928; Jones et al., 2001; Liu et al., 2008). Several mechanisms underlie the limited regenerative capacity of the adult CNS,

including factors both intrinsic and extrinsic to the neuron. Intrinsically, many injured adult neurons fail to express a set of genes that are required to enter a state of active growth (Woolf et al., 2001; Bomze et al., 2001; Cafferty et al., 2004; Mason et al., 2002). Simultaneously, the environment of the injured adult CNS lacks growth factors to stimulate growth (Tuszynski et al, 1996; Bradbury et al., 1999; Liu et al., 1999a), and expresses inhibitory molecules both in the extracellular matrix (Filbin, 2003; Silver 2004) and in adult myelin that limit axonal plasticity (Schwab 1993 ,Löw 2008). By addressing each of these mechanisms individually, considerable progress has been made in enhancing the growth potential of injured adult axons (Yick et al., 2003; Davies et al., 2004; Kim et al., 2006; Caroni et al., 1997; Kelley et al., 1997; Neuman et al., 2002; Qiu et al., 2002; Spencer et al., 2004; Schnell et al., 1994; Tetzlaff et al., 1994; Xu et al., 1995; Grill et al., 1997; Kobayashi et al., 1997; Ye et al., 1997; Jakeman et al., 1998; Liu et al., 1999b; Blits et al., 2000; Ramer et al., 2000; Bamber et al., 2001; Coumans et al., 2001; Himes et al., 2001; Lu et al., 2001; Plunet et al., 2002). Further, when combinatorial treatments are applied, the first reliable indications that axons can be induced to regenerate beyond sites of spinal cord injury have recently appeared (Lu et al., 2004; Houle et al., 2006; Massey et al., 2008).

Many experimental approaches to promote axonal regeneration utilize cell suspension grafts into sites of injury that support axonal attachment and elongation, including fibroblasts, astrocytes, Schwann cells, olfactory ensheathing cells or bone marrow stromal cells (Pizzi et al., 2006; Jin et al., 2002; Franklin et al., 1991; Cunningham et al., 1991; Agudo et al., 2006; Golden et al., 2007; Franssen et al.,

2007; Plant et al., 2003; Shichinohe et al., 2008; Lu et al., 2005). However, a drawback of cellular implants is their lack of 3-dimensional organization, typically resulting in random directions of axonal growth in the lesion; as a result, relatively few axons reach the distal aspect of a lesion site, and few are then available to recruit for bridging beyond the lesion (Lu et al., 2004). Bioengineered scaffolds offer the potential advantage of enhancing the organization of axon growth into injury sites (Moore et al., 2006; Hurtado et al., 2006; Wong et al., 2008). In concept, scaffolds should be strictly linear to retain the native fascicular organization of axonal projections in the spinal cord. Scaffolds such as poly-lactic glycolytic acid (PLGA), alginate and collagen can retain high degrees of linearity, but over distances approximating the length of human spinal cord injures, 2-4 cm, the linearity of channels in these scaffold types appears vulnerable to degradation and formation of side-channels that corrupt linear architecture. For this reason, we fabricated strictly linear guidance channels using fiber templating technology (Stokols et al., 2006). Adapting fabrication technology from fiber optic bundle manufacturing, we generated templated agarose scaffolds of strictly linear configuration over distances of at least 10 mm, with a honeycomb architecture and individual channels of 200μm diameter (Stokols 2006). When grafted in vivo to sites of rodent spinal cord injury, templated agarose scaffolds supported linear growth of local spinal cord axonal populations (Stokols 2006).

In the present set of studies, we determined whether templated agarose scaffolds would further support linear growth of long-tract axons of the injured adult spinal

cord, and support axonal bridging beyond sites of injury when combined with therapies to promote axonal egress from lesion sites. Accordingly, we previously reported that long-tract sensory axons of the spinal cord dorsal columns can regenerate beyond sites of spinal cord injury following the application of combinatorial therapies, consisting of: 1) autologous bone marrow stromal cells grafted into the lesion site; 2) injections of lentiviral vectors expressing the sensory axon growth factor neurotrophin-3 beyond the lesion site, to provide a chemotropic gradient to attract axons beyond the lesion site (Taylor 2006), and 3) “conditioning” compression lesions of the sciatic nerve, to upregulate intrinsic neuronal gene expression and activate the growth of the central sensory axon (Reich et al., 1990; Hannila et al., 2008; Nueman et al., 1999; Lu et al., 1991). The combined treatment promoted axon growth into and beyond these cell suspension grafts in the lesion site, even though few axons reached the distal aspect of the lesion cavity due to the random orientation of growth through the non-organized graft (Lu et al., 2004). Notably, axons only regenerated when the full treatment combination was applied; provision of only the cell graft and trophic factors, or cell graft and conditioning lesion, did not achieve bridging. We therefore examined in the current experiment whether the combination of a templated agarose scaffold, an NT-3 gradient beyond the lesion, and a conditioning lesion would organize and enhance axonal bridging through sites of spinal cord injury. Comparison was made to scaffold-implanted subjects lacking the full combination treatment, and to animals that received cell suspension grafts into the lesion cavity instead of templated agarose scaffolds.

Materials and methods

Experimental design:

Six groups of animals were studied. All groups underwent C4 spinal cord dorsal column lesions, as described below. Group 1 (n=6) received implants of templated agarose scaffolds into the lesion cavity (scaffolds were pre-seeded with autologous marrow stromal cells that secreted NT-3), injections of lentiviral vectors expressing NT-3 1.5 mm beyond (rostral to) the C4 lesion site (to elicit axonal egress from scaffolds), and conditioning of the sciatic nerve (transient compression to induce a growth state in the injured neuron). Group 2 (n=6) received implants of templated agarose scaffolds into the lesion cavity (pre-seeded with NT-3-secreting stromal cells), and injections of lentiviral vectors expressing NT-3 1.5 mm beyond the C4 lesion site; sham surgery was performed on the sciatic nerve but it was not compressed. Group 3 (n=6) received implants of templated agarose scaffolds into the lesion cavity (pre-seeded with NT-3-secreting stromal cells) and conditioning of the sciatic nerve, and lentiviral vectors expressing only the reporter Green Fluorescent Protein (GFP) were injected beyond the lesion site. Thus, a growth factor gradient beyond the lesion was not provided. Group 4 (n=6) received implantations of templated scaffolds only (pre-seeded with NT-3-secreting stromal cells), with control vectors injected beyond the lesion and sham sciatic nerve surgery. Group 5 (n=6) received implantations of empty (non-seeded) templated scaffolds only, with control vectors injected beyond the lesion

and sham sciatic nerve surgery. Group 6 (n=6) received full combinatorial therapy as in Group 1, but instead of receiving implants of templated scaffolds, this group received cell suspension grafts of NT-3-secreting cells into the lesion cavity (a non-organized cellular matrix). Subjects survived for 4 weeks after lesions and treatments.

Fabrication and sterilization of scaffolds:

Multi-component fiber bundles (MCFBs) were used to generate precise linear channels, as previously described (Fig. 2.1; Stokols 2006). Briefly, to generate scaffolds with precise linear channels, multi-component fiber bundles (MCFBs) were manufactured from 200-mm-diameter polystyrene fibers (Paradigm Optics, Vancouver, WA). Fibers were arranged in a hexagonal close-packed array separated by a continuous matrix of poly(methyl methacrylate) (PMMA). MCFBs were simultaneously extruded and fused such that the polystyrene fibers were oriented parallel to the longitudinal axis of the bundles. The MCFBs were trimmed to 2 mm longitudinal length and cross-sectional diameter of 1.5 mm. Polystyrene end caps of 1.5 mm length were bonded to the ends of the MCFBs using a 1:1 acetone/toluene (v/v) mixture to anchor polystyrene fibers within the MCFB to an external, rigid material. Next, polystyrene side caps were bonded to the ends of MCFBs. The PMMA matrix was then selectively removed by immersing MCFB templates in 99.7% propylene carbonate (Sigma-Aldrich) at 45^{o}C for 24 hr; this was repeated 3 times to ensure complete removal of PMMA. The templates were then washed in 95% ethanol and distilled water prior to agarose casting.

Ultrapure agarose (30 mg/mL; Sigma-Aldrich) was dissolved in distilled water at 100^{o}C. After cooling of agarose to 65^{o}C, MCFB templates were submerged into the agarose solution and centrifuged at 300rpm for 20 sec to overcome surface tension between the agarose and closely packed polystyrene fibers. After maintenance at 65^{o}C for 5 min, the heat source was removed and agarose was allowed to gel at room temperature. MCFB templates, now permeated with gelled agarose, were cut from the gelled agarose aggregate, leaving just an agarose scaffold cast, bound by polystyrene caps and fiber mold. The polystyrene fibers, end-caps, and side-caps were dissolved by immersing templates in 99% tetrahydrofuran (THF) (Sigma-Aldrich) at room temperature for 24 hr. This process was repeated 3 times to ensure complete removal of polystyrene, resulting in individual free-floating agarose scaffolds. Scaffolds were collected and washed in acetone, then stored in 95% ethanol for sterile storage until future use. One day before implantation the scaffolds were washed in 3 cycles of autoclaved distilled water and stored in a sterile UV hood until implantation.

Analysis of scaffold architecture

Scaffold microstructure was visualized under phase-contrast microscopic optics, as previously described (Stokols 2006). Pore dimensions and porosity were directly measured from light micrographs. Templated agarose scaffolds with channel diameters of 200μm and a uniform wall thickness of 100μm were consistently produced (Fig. 2.1). Scaffolds contained uniaxial channels organized in an array precisely replicating the pattern of polystyrene fiber cores in the MCFB. Scaffold

microstructure was also examined under light level microscopy, and the rare scaffold with imperfections of channel architecture was not used for in vivo studies.

Production of lentiviral vectors

The vector pLV (Pfeifer 2002) was used to construct NT-3-secreting and control (green fluorescent protein (GFP)-expressing) vectors as described (Taylor 2006). NT-3 or GFP expression was driven by the cytomegalovirus/-actin hybrid promoter (CAG) (Niwa 1991). NT-3 expressing virus also expresses GFP as reporter gene from an internal ribosome entry site.

Third-generation lentiviral vector plasmids with a split genome packaging system were used for the production of human immunodeficiency virus (HIV) vectors as described previously (Naldini et al., 1996; Blesch et al., 2004). High-titer stocks of HIV vectors were prepared by ultracentrifugation. Titers of GFP-expressing virus were determined by infection of HEK293T cells using serial dilutions. After 48 hr, GFP-expressing colonies were quantified for each dilution to determine infectious units (IU) per milliliter. Vector stocks were also assayed for p24 antigen levels using an HIV-1 p24-specific ELISA kit (DuPont, Billerica, MA) as described previously (Naldini 1996). NT-3 vector preparations contained 112-134ug/ml p24 and $4x10^7$ to $1.5x10^8$ IU/ml. Control vector preparations contained 95-112ug/ml p24 and $1\text{-}5x10^8$ IU/ml.

Isolation of bone marrow stromal cells

Rat primary bone marrow stromal cells (MSCs) were isolated according to the method of (Azizi et al., 1998). Briefly, Fischer 344 adult female rats were anesthetized with a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 mg/ml), and acepromazine (0.25 mg/ml) and decapitated, and tibias and femurs were dissected. After removing the end of each bone, 5 ml of alpha-MEM (Invitrogen, Carlsbad, CA) was injected into the central canal of the bone to extrude marrow. Cells were cultured in D'MEM (Invitrogen) supplemented with 20% fetal bovine serum and antibiotics. Nonadherent cells were removed after 24 h. Cells were passaged twice and either frozen or transduced with Moloney murine leukemia virus-based retroviral vectors for the expression of NT-3 (Grill 1997). Before grafting, cells were thawed and cultured in the same media as above.

Transduction of marrow stromal cells with retroviral vectors to express NT-3

MSCs were transducted with retroviral vectors before grafting into scaffolds. Syngenic MSCs were genetically modified to produce and secrete human NT-3 using a stable PA317 retrovirus producer cell line, as described previously (Grill 1997). Conditioned media from producer cultures was used to infect MSCs, and transduced cells were selected for G418 resistance (100 *u*g/ml) for 11 days. After selection, cells were grown to confluency, and 24 hr supernatants were collected for ELISA of NT-3 protein expression (NT-3 Emax ImmunoAssay System; Promega, Madison, WI).

Transduced MSCs produced 165 ng of NT-3/10^6cells/24hr. Cells were resuspended to a final concentration of 75,000 cells/µl.

Lesion surgery and vector injection

Adult female Fischer 344 rats (n=60) weighing 150-200 g were used. Institutional guidelines on animal care were strictly followed. Under deep anesthesia, ketamine cocktail (ketamine (50 mg/kg), xylazene (2.6 mg/kg) and acepromazine (0.50 mg/kg) , the C4 lamina was removed, and a rectangular lesion of rostral-to-caudal length 2 mm, width 1.5 mm (centered at midline), and depth 1.5 mm ventrally was created at C4 using microscissors and microaspiration. Scaffolds were carefully placed into the lesion cavity in a manner to achieve a close ‘‘fit’’ between the rostral and caudal portions of the spinal cord surrounding the scaffold. If the scaffold fit loosely or was excessively compressed within the lesion, it was replaced. Immediately prior to surgery, 2 µl (75,000 cells/µl) of MSC, MSC–NT3 or PBS were pre-loaded into the scaffolds. Following implantation of the scaffold, 3% agarose film was placed over the implant site. Next, lentiviral vectors (2.5 µl volume) were injected through pulled glass capillaries into the spinal cord 1.5mm rostral to the lesion, in the spinal cord midline, at a depth of 0.5 and 1 mm. Injections were made at a rate of 0.5 *u*l/min (1.25 µl at each depth), using a Picospritzer II (General Valve, Fairfield, NJ). Pipettes were left in place for 1 min after the injection and were then slowly withdrawn. Overlying muscle layers were sutured and the skin was stapled. The next day, subjects underwent sciatic nerve conditioning or sham surgery. Following exposure of the

sciatic nerve at mid-thigh, the sciatic nerve was compressed with firm and steady pressure for 15 sec with a #5 Dumont jeweler's forceps, twice. Animals survived an additional 4 weeks. Ascending sensory projections were then labeled by injections of the transganglionic tracer cholera toxin B subunit (CTB) into the sciatic nerve, 72 hours prior to sacrifice (injection of 2 ul of a 1% CTB solution; List Biologic, Campbell, CA).

Immunohistochemical labeling

Animals were transcardially perfused with 100ml of cold phosphate buffered saline (PBS), followed by 300 ml of 4% paraformaldehyde in phosphate buffer. Spinal cords and brainstems were removed, postfixed overnight in 4% paraformaldehyde, and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose at 4°C. Spinal cords were blocked in TBS Tissue Freezing Media (Triangle Biomedical Sciences) on dry ice, and sagittal sections were cut on a freezing microtome set at 35 µm intervals.

Spinal cord sections were labeled for CTB using streptavidin-HRP light-level immunohistochemistry, followed by GFP and glial fibrillary acidic protein (GFAP) fluorescent immunolabeling on the same sections (triple labeling). The following immunocytochemical markers were used: goat anti-CTB (1:50,000; List Biologic) to detect ascending sensory axons; GFAP (monoclonal, 1:1000; Chemicon, Temecula, CA) to label astrocytes; and rabbit anti-GFP (1: 1000; Invitrogen) to label vector-transduced cells. Immunocytochemical labeling for CTB was performed first with streptavidin–HRP-based light-level immunohistochemistry using free-floating sections

in the following protocol: (1) overnight incubation in primary antibodies at 4°C; (2) incubation for 2 h with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA) at 4°C; (3) 1 h incubation with avidin-biotinylated peroxidase complex (1:150; Vector Elite kit; Vector Laboratories) at room temperature; and (4) treatment for 10 min with 0.05% solution of 3,3-diaminobenzidine, 0.01% H2O2, and 0.04% nickel chloride at room temperature. Fluorescence immunohistochemistry for GFP and GFAP was then performed on the same sections according to the following protocol: (1) overnight incubation in primary antibody; and then (2) 4 hr incubation with fluorescent-conjugated secondary antibodies (donkey anti-rabbit Alexa-488 at 1:200; donkey anti-mouse Alexa 594 at 1:200; Invitrogen). Labeled sections were mounted and coverslipped with a methacrylate polymer (Pro-Texx, Lerner Laboratories, Pittsburg, PA).

Additional sections were counterstained (Nissl, cresyl violet acetate, Polysciences, Inc.), or immunolabeled for ionized calcium binding adapter molecule (IBA-1, 1:1000, Wako), or macrophages (ED1, 1:1000, Chemicon), condroiten proteoglycan (NG2, 1:200, Abcam). Free-floating sections (see above) were placed in TBS-Triton 0.25% for 3x10 minutes and then in a serum blocking solution of 4% donkey serum with TBS-Triton 0.25% for 3 hrs. Next, they were washed in TBS for 3x10minutes and placed in primary antibody solution overnight. The next day, sections were washed for 3x10mintes and placed in a secondary antibody for 5 hrs (6:1000 Chemicon 488 and 594 anti- goat, mouse, or rabbit, respectively).

Assessment and quantification of axon density in scaffolds

To determine the number of CTB-labeled axons entering and exiting scaffolds, every 8th 35µm-thick sagittal spinal cord section triple labeled for CTB, GFAP, and GFP was examined by an observer blinded to group identity. The number of individually identifiable CTB-labeled axons crossing a vertical line at 200µm intervals in every scaffold was quantified, using a reticle eyepiece at 200x mangification. Care was taken not to double-count individual axons. Mean axon number in each region was compared using ANOVA, and post-hoc comparisons were made using Fisher's t-test with a significance criterion of $p<0.05$. Similar methods of quantification were used to measure axon density within the group of subjects receiving implants of cell suspension grafts (Group 6), sampling the caudal, middle and rostral aspects of the graft in each subject.

Quantification of cell orientation within scaffolds vs. cell suspension grafts

To compare cellular orientation within scaffolds or cell suspension grafts, Nissl-stained cells were examined under 400x magnification over the full scaffold or graft length in Groups 1 and 6 (combinatorial scaffold group and combinatorial cell suspension group, respectively, measuring 6 animals/group). For each animal, three randomly sampled images within the lesion site were acquired using PictureFrame and imported to ImageJ for measurement of cellular orientation (angle of orientation of long axis of cell relative to the longitudinal axis of the spinal cord) in Nissl stained

sections (Fig. 2.3). Cells with an elliptical shape were examined, and the angle of orientation of the two ends of the ellipse were measured with reference to the angle of channel walls, or, in cell suspension graft animals, to the angle of the longitudinal axis of the spinal cord . Three sections were analyzed per subject. A mean distribution of cell orientation within 360^{o} space was calculated for each group. Distributions were compared using ANOVA and post-hoc Fisher's, using a significance criterion of $p<0.05$.

Quantification of sensory axon orientation within scaffolds vs. cell suspension grafts

To compare sensory axon orientation within scaffolds or cell suspension grafts, CTB-labeled axons were examined under 400x magnification over the full scaffold or graft length in Groups 1 and 6 (combinatorial scaffold group and combinatorial cell suspension group, respectively, measuring 6 animals/group). For each animal, three randomly sampled images within the lesion site were acquired using PictureFrame and imported to ImageJ for measurement of axon orientation (angle of orientation of axon relative to the longitudinal axis of the spinal cord) in CTB stained sections (Fig. 2.3). Axons were approximated as 100um lines, and the angle of orientation of the axon segment were measured with reference to the angle of channel walls, or, in cell suspension graft animals, to the longitudinal axis of the spinal cord. Three sections were analyzed per subject. A mean distribution of axon orientation within 360^{o} space was calculated for each group. Distributions were compared using ANOVA and post-

hoc Fisher's, using a significance criterion of $p<0.05$.

Quantification of GFP and microglial cell density

To determine the density of GFP-transduced cells, and host microglial cells penetrating agarose scaffolds, a series of 1-in-8 sections was labeled for each marker. Two sections from this series per animal that were most centrally located within each scaffold were then selected for quantification by an observer blinded to group identity. Each label was quantified within a 200μm box located at the caudal, middle and distal aspect of each scaffold. GFP-labeled cells and IBA-1-labeled microliga and macrophages were quantified at 200x magnification. Images of each region were captured using PictureFrame software (Optronics, Goleta CA), using the same filter, laser intensity, and shutter settings on each subject, on an Olympus AX70 microscope. The number of pixels of each labeled reaction product was determined and mean values per subject per label were generated. Findings between groups were compared using ANOVA, with post-hoc Fisher's using a significance criterion of $p<0.05$.

Ultrastructural examination

Three animals that received scaffolds and full combinatorial therapies were examined at the ultrastructural level. Subjects were perfused with Millinog perfusion buffer and spinal cords were stored in glycerin. Representative sections were stained with Toluidine Blue for morphology or gold sputtering for electron microscopic imaging. All myelinated axons per channel were quantified in six randomly sampled

channels per animal at 400x magnification in toluidine blue-stained sections; the mean number of axons per channel was then multiplied by the total number of channels per subject to generate an estimate of the mean number of axons per scaffold.

Statistics:

Statistical methods are noted in individual sections above. All analyses were conducted by an investigator blinded to group identity.

Results

Combinatorial treatments support regeneration of long tract, ascending dorsal column sensory axons into and beyond the scaffold implant

Six groups of subjects were examined in this study, including animals that received combinatorial treatment consisting of templated agarose scaffolds, trophic factor gradients and conditioning lesions of dorsal root ganglia neurons (Fig. 2.1). Among these groups, significantly greater numbers of sensory axons regenerated into and beyond scaffolds in full combinatorially-treated subjects (Figs. 2.2, 2.3, 2.4). Subjects that received scaffolds plus growth factor gradients (but no conditioning lesions), or scaffolds plus conditioning lesions (but no growth factor gradients), exhibited fewer axons regenerating over the full length of scaffolds than combinatorially-treated animals ($p<0.05$; Fig. 2.4). Subjects lacking either conditioning lesions or growth factor gradients exhibited the fewest number of

regenerating axons (Fig. 2.4). Axons that regenerated into agarose channels in all subjects exhibited strikingly linear orientations along the caudal-to-rostral plane of the spinal cord (Fig. 2.2). Scaffold implants retained their pre-implant 3-dimensional honeycomb architecture and shape throughout the one-month in vivo portion of this experiment, without evident degradation over this time period (Fig. 2.1).

Combinatorial treatments enhance the total number and proportion of axons reaching the distal end of a spinal cord lesion cavity, compared to randomly oriented cell suspension grafts

A central hypothesis of this experiment was that the three-dimensional organization of the regeneration milieu would guide regenerating axons linearly over the full length of the lesion cavity. To test this hypothesis, we compared the number and proportion of CTB-labeled sensory axons reaching the distal aspect of the lesion cavity in combinatorially treated subjects that received scaffold implants to combinatorially treated subjects that received randomly oriented cell suspension grafts (Fig. 2.2). A highly significant increase in the number of sensory axons regenerating to the distal aspect of the lesion cavity was observed in scaffold-implanted subjects (Fig. 2.5: $p<0.01$). 83 $\pm$ 13% of sensory axons entering scaffolds reached the distal end of the lesion cavity; in contrast, only 22 $\pm$ 6% of axons penetrating non-organized, cell suspension graft lesion cavities regenerated to the end of the lesion site. A 2^{nd} degree polynomial function described the efficiency of axonal retention over distance in scaffold-implanted subjects: $y = 0.04x^2 - 0.16x + 0.99$ (Chi-squared = 0.95; x

represents distance from caudal edge of lesion, y represents remaining fraction of CTB population). In contrast, cell suspension grafts were described by a function of substantially lower magnitude: $y = 0.23x^2 - 0.86x + 1.01$ (Chi squared 0.99). . Thus, combinatorial therapies applied in conjunction with templated scaffolds significantly increase the number and proportion of regenerating axons that reach the end of a spinal cord lesion site, thereby enhancing the pool of axons that are potentially available to continue growth in the host spinal cord located beyond the lesion site.

The cellular substrate and sensory axons are linearized within the scaffold implant

To examine potential mechanisms underlying the linear organization of axonal growth through the scaffold environment, we examined and quantified markers of cell types penetrating scaffolds. Using a general cell marker for Nissl substance (thionin stain), 80 $\pm$ 5% of cells within scaffolds were linearly arranged (90 $\pm$ 5 degrees) along the rostral-to-caudal axis of the spinal cord, whereas only 6 $\pm$ 5% of sampled cells were oriented in the rostral-to-caudal axis of the spinal cord in subjects that received randomly organized cell suspension grafts (Fig. 2.6). Additionally, we examined the orientation of axonal growth through the scaffold environment. Examining CTB-labeled axons, 36 $\pm$ 5% of axons within scaffolds were linearly arranged (90 $\pm$ 5 degrees) along the rostral-to-caudal axis of the spinal cord, whereas only 6 $\pm$ 5% of the axons were oriented in the rostral-to-caudal axis of the spinal cord in subjects that received randomly organized cell suspension grafts. When considering a broader range of linearity (90 $\pm$ 15 degrees) there were 84 $\pm$ 5% and 18 $\pm$ 5% CTB labeled axons in

scaffold and cell suspension groups, respectively (Fig. 2.6). Thus, cells were constrained by the cylindrical three-dimensional structure of scaffolds to orient along the longitudinal axis of the scaffold; axons extend their processes along the surfaces of cells within lesion environments, thereby presumably conforming to the longitudinal orientation of the cell surfaces to grow in linear planes (Weidner 1999, Pfeifer 2004). This linear orientation was absent in cell suspension grafts (Fig. 2.6).

In addition, labeling for the reporter gene GFP indicated that injections of lentiviral NT-3-expressing vectors often extended into scaffolds (Fig. 2.7B); concentrations of NT-3 are elevated within these regions of gene expression (Taylor 2006), thereby enhancing the protein gradient of NT-3 within the scaffold environment to attract axons (Fig. 2.S1). As previously reported (Stokols 2006), only rare inflammatory cells were present within scaffolds, reflected by astrocyte (GFAP) and microglial (IBA) immunolabeling (Fig. 2.7, 2.S2).

Electron microscopy confirmed the presence of numerous axons within scaffold implants (Fig. 2.7). Quantification revealed a mean number of 340 $\pm$ 20 axon per channel, corresponding to a total of 8,500 axons per scaffold (each implanted scaffold contained 25 channels). Axons within implants were present in both myelinated and unmyelinated bundles; myelinating cells were Schwann cells, evidenced by the close juxtaposition of the Schwann cell nucleus to the associated axonal process (Fig. 2.7). Cells with ultrastructural features of oligodendrocytes were never observed within scaffolds. Similarly, astroctyes were not observed within scaffolds, consistent with the lack of GFAP immunolabeling. Blood vessels were

readily detectable within scaffolds, at a density of 8 ± 2 vascular profiles per 0.03mm^2 cross-sectional sample. This corresponds to a density of one vessel per 400μm^2, a sufficient density to support molecular diffusion from vasculature to cells in the spinal cord (Imperato-Kalmar 1997).

Regenerating sensory axons do not re-penetrate host spinal cord beyond the lesion

While axons readily penetrated and subsequently regenerated beyond scaffolds in combinatorially-treated subjects, axons did not re-penetrate host parenchyma beyond the lesion. Instead, axons filled a host cell layer that was interposed between the distal aspect of the scaffold implant and distal spinal cord. This "reactive" cell layer was of 150 ± 100μm mean width (from end of scaffold to host spinal cord). Ultrastructural analysis indicated that the reactive cell layer consisted primarily of cells with features of leptomeningeal fibroblasts (Fig. 2.8). The border between the reactive cell layer and host parenchyma was demarcated by GFAP immunoreactivity (Fig. 2.8). Further, the inhibitory extracellular matrix marker NG2 exhibited a distinct border of labeling at the reactive cell layer/host parenchymal interface (Fig.2.8). Notably, the reactive cell layer was present at both ends of the scaffold implant, but did not prevent CTB-labeled sensory axon penetration into scaffolds. However, at the rostral end of the scaffold, axons did not regenerate *beyond* the reactive cell layer to repenetrate host gray or white matter, a finding that may be a function of the time at which the reactive cell layer forms (discussed below). The reactive cell layer did not form in subjects that underwent combinatorial therapies with cell suspension grafts

instead of scaffold implants into the lesion cavity. Notably, while the total number of axons reaching the distal aspect of the lesion cavity was 60% lower in subjects with cell suspension implants compared to scaffold-implanted animals, CTB-labeled sensory axons nonetheless re-penetrated host spinal cord parenchyma beyond the lesion in subjects with cell suspension grafts but not in subjects with scaffold implants, consistent with previous reports using combination treatments with cell suspension grafts (Lu et al., 2004). Thus, the reactive cell layer that forms between host spinal cord parenchyma and the scaffold supports axonal emergence from scaffolds, but axons do not regenerate beyond this cell region.

Discussion

Microstructure is important in directing cell migration, attachment, and orientation (Palsson, 2004; Ratner, 2004), creating mechanical and molecular stresses that can affect cellular morphology and function (Fung, 1990; De et al., 2007; Langer, 1993). The linearized microstructure design of our agarose scaffolds potentially minimizes the amount of non-uniform stress upon cellular migration, matrix deposition, and cell grafting, thereby creating linear, non-tortuous tissue architecture. Indeed, we find that templated agarose scaffolds can linearly guide long-tract axons completely through a spinal cord lesion site, resulting in a 4-fold increase in the number of axons reaching the distal aspect of a lesion cavity compared to a non-organized cell suspension graft. This represents a substantial advance over existing

cell suspension technologies commonly utilized in the neural repair field. These regenerating axons emerge from scaffolds and extend into a cellular matrix that intercalates between the agarose implant and host spinal cord tissue; however, the regenerating axons do not re-penetrate the host spinal cord beyond the lesion site. Thus, while the present findings represent an advance over existing cellular methodologies in enhancing axonal regeneration through a spinal cord lesion site, they also clearly identify the next challenge in continued development of this technology: reduction of the reactive cell matrix that forms between the scaffold and host parenchyma.

The nature of the cellular matrix forming between the scaffold implant and host spinal cord was identified by electron miscroscopic analysis: these cells are primarily fibroblastic in nature, likely migrating from the overlying leptomeninges. The extracellular matrix these cells form contains collagen, potentially trapping axons and preventing their re-penetration of the host spinal cord. The reactive cell matrix did not contain large numbers of inflammatory cells by Nissl stain, or by the specific cellular labels GFAP and IBA1 (for astrocytes and microglia, respectively). There are several potential approaches to reducing the reactive cell response between scaffold and host, as a means of facilitating axonal regeneration into host spinal cord beyond the lesion: 1) Delay implantation of scaffolds for several days after injury, until fibroblast/leptomeningeal cellular activation resulting from the traumatic injury are reduced (Ross et al., 1968; Conrad et al., 2005; Popovich et al., 1997). 2) Place a matrix barrier between the scaffold and leptomeningeal cell layer. For example, a thin

layer of agarose film or a fibrin matrix (e.g., Tisseal^{R}) may impede the migration of leptomeningeal cells into the lesion site. 3) Apply anti-mitotic agents to reduce the division and subsequent migration of leptomeningeal cells or fibroblasts into the site of scaffold implantation (Pruss et al., 1979; Brockes et al., 1979). We are currently examining these approaches in in vivo models.

Findings of the present study confirm previous reports that combinatorial targeting of several mechanisms that ordinarily limit axonal regeneration can maximize axonal growth in models of CNS injury (Lu et al., 2004; Pearse et al., 2004; Nikulina et al., 2004; Houle et al., 2007; Fisher et al., 2004). For example, in previous studies we found that sensory axonal growth into and beyond a mid-cervical site of spinal cord injury is maximized when the intrinsic neuronal growth program is stimulated by cAMP or "conditioning" lesions of the sciatic nerve, and when the environment of the injured axon is enhanced by providing a neurotrophic stimulus to attract the injured axon beyond the injury (Lu 2004). Similarly, in the present study the combined stimulation of the injured sensory neuron by a conditioning lesion, and the provision of a trophic gradient beyond the lesion, maximized the number and proportion of axons extending beyond the scaffold implant site compared to groups that did not receive the full combination therapy. Indeed, the provision of an organized growth scaffold in the lesion site further increased the number and proportion of axons reaching the distal aspect of the lesion site in combinatorially-treated subjects, compared to combinatorially-treated subjects that received non-organized cell suspension grafts in the lesion site. However, in combinatorially-treated subjects that

received cell suspension grafts, a reactive cellular matrix does not form between the cell implant and host spinal cord tissue, and axons successfully continue to regenerate into host spinal cord located beyond the lesion (Lu 2004). Thus, it is essential that further development of the scaffold technology focus on limiting the reactive cell matrix that forms between the scaffold and host spinal cord, to determine whether the greater pool of axons presented to the distal aspect of the lesion results in a greater number of axons regenerating into the host spinal cord beyond the lesion site.

Acknowledgement

Chapter 2 is original work in preparation as Gros TR, Sakamoto J, Blesch A, Havton L, Tuszynksi MH. Templated agarose scaffolds orient and guide regenerating long-tract axons through sites of spinal cord injury and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

References

Agudo, M, A Woodhoo, D Webber, R Mirsky, K Jessen and S Mcmahon (2008) Schwann cell precursors transplanted into the injured spinal cord multiply, integrate and are permissive for axon growth. Glia.

Azizi, S, D Stokes, B Augelli, C Digirolamo and D Prockop (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats-similarities to astrocyte grafts. Proceedings of the National Academy of Sciences 95(7): 3908-13.

Azizi, S, D Stokes, B Augelli, C Digirolamo and D Prockop (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats-similarities to astrocyte grafts. Proceedings of the National Academy of Sciences 95(7): 3908-13.

Baekelandt, V, A Claeys, K Eggermont, E Lauwers, B De Strooper, B Nuttin and Z Debyser (2002) Characterization of lentiviral vector-mediated gene transfer in adult mouse brain. Human Gene Therapy 13(7): 841-53.

Bamber, N, H Li, X Lu, M Oudega, P Aebischer and X Xu (2001) Neurotrophins bdnf and nt-3 promote axonal re-entry into the distal host spinal cord through schwann cell-seeded mini-channels. European Journal of Neuroscience 13(2): 257-68.

Blesch, A (2004) Lentiviral and mlv based retroviral vectors for ex vivo and in vivo gene transfer. Methods 33(2): 164-72.

Blesch, A, H Yang, N Weidner, A Hoang and D Otero (2004) Axonal responses to cellularly delivered nt-4/5 after spinal cord injury. Molecular and Cellular Neuroscience 27(2): 190-201.

Blits, B, P Dijkhuizen, G Boer and J Verhaagen (2000) Intercostal nerve implants transduced with an adenoviral vector encoding neurotrophin-3 promote regrowth of injured rat corticospinal tract fibers and improve hindlimb function. Experimental Neurology 164(1): 25-37.

Bomze, H, K Bulsara, B Iskandar, P Caroni and J Skene (2001) Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nature Neuroscience 4: 38-43.

Bradbury, E, S Khemani, R Von, J Priestley and S Mcmahon (1999) Nt-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. European Journal of Neuroscience 11(11): 3873-83.

Brockes, J, K Fields and M Raff (1979) Studies on cultured rat schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res 165(1): 105-18.

Cafferty, W, N Gardiner, I Gavazzi, J Powell, S Mcmahon, J Heath, J Munson, J Cohen and S Thompson (2001) Leukemia inhibitory factor determines the growth status of injured adult sensory neurons. Journal of Neuroscience 21(18): 7161.

Cafferty, W, N Gardiner, P Das, J Qiu, S Mcmahon and S Thompson (2004) Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. Journal of Neuroscience 24(18): 4432.

Caroni, P (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. BioEssays 19(9): 767-75.

Caroni, P (1998) Neuro-regeneration: Plasticity for repair and adaptation. Essays Biochem 33: 53-64.

Carulli, D, T Laabs, H Geller and J Fawcett (2005) Chondroitin sulfate proteoglycans in neural development and regeneration. Current Opinion in Neurobiology 15(2): 252-52.

Chen, H and E Frank (1999) Development and specification of muscle sensory neurons. Current Opinion in Neurobiology 9(4): 405-09.

Chotard-Ghodsnia, R, A Drochon, N Faucheux, M Nagel and R Grebe (2002) Effect of shear stress and of transmural pressure on camp-dependent responses of cells adhering to a biomaterial. Eur. Phys. J. AP 17: 155-62.

Coumans, J, T Lin, H Dai, L Macarthur, M Mcatee, C Nash and B Bregman (2001) Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. Journal of Neuroscience 21(23): 9334.

Cunningham, L, J Hansen, M Short and M Bohn (1991) The use of genetically altered astrocytes to provide nerve growth factor to adrenal chromaffin cells grafted into the striatum. Brain Res 561(2): 192-202.

Davies, J, X Tang, J Denning, S Archibald and S Davies (2004) Decorin suppresses neurocan, brevican, phosphacan and ng2 expression and promotes axon growth across adult rat spinal cord injuries. European Journal of Neuroscience 19(5): 1226-42.

De, R, A Zemel and S Safran (2007) Dynamics of cell orientation. Nature Physics 3(9): 655.

Dobkin, B (2003). The clinical science of neurologic rehabilitation, Oxford University Press, USA.

Dontchev, V and P Letourneau (2002) Nerve growth factor and semaphorin 3a signaling pathways interact in regulating sensory neuronal growth cone motility. Journal of Neuroscience 22(15): 6659.

Ernfors, P and H Persson (1991) Developmentally regulated expression of hdnf/nt-3 mrna in rat spinal cord motoneurons and expression of bdnf mrna in embryonic dorsal root ganglion. European Journal of Neuroscience 3(10): 953-61.

Farinas, I, C Yoshida, C Backus and L Reichardt (1996) Lack of neurotrophin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron(Cambridge, Mass.) 17(6): 1065-78.

Filbin, M (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian cns. Nature Reviews Neuroscience 4(9): 703-13.

Fischer, D, V Petkova, S Thanos and L Benowitz (2004) Switching mature retinal ganglion cells to a robust growth state in vivo: Gene expression and synergy with rhoa inactivation. Journal of Neuroscience 24(40): 8726-40.

Förander, P, L Björklung and I Strömberg (1994) Dose-dependent effects of recombinant human ngf on grafted adult adrenal medullary tissue. Experimental neurology(Print) 126(2): 168-77.

Franklin, R, A Crang and W Blakemore (1991) Transplanted type-1 astrocytes facilitate repair of demyelinating lesions by host oligodendrocytes in adult rat spinal cord. Journal of Neurocytology 20(5): 420-30.

Franssen, E, F De Bree and J Verhaagen (2007) Olfactory ensheathing glia: Their contribution to primary olfactory nervous system regeneration and their regenerative potential following transplantation into the injured spinal cord. Brain Research Reviews 56(1): 236-58.

Fung, Y (1993). Biomechanics: Mechanical properties of living tissues, Springer.

Gallo, G, F Lefcort and P Letourneau (1997) The trka receptor mediates growth cone turning toward a localized source of nerve growth factor. Journal of Neuroscience 17(14): 5445-54.

Gao, Y, E Nikulina, W Mellado and M Filbin (2003) Neurotrophins elevate camp to reach a threshold required to overcome inhibition by mag through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase. Journal of Neuroscience 23(37): 11770-77.

Genç, B, P Özdinler, A Mendoza and R Erzurumlu (2004) A chemoattractant role for nt-3 in proprioceptive axon guidance. PLoS Biol 2(12): e403.

Golden, K, D Pearse, B Blits, M Garg, M Oudega, P Wood and M Bunge (2007) Transduced schwann cells promote axon growth and myelination after spinal cord injury. Experimental Neurology 207(2): 203-17.

Grill, R, K Murai, A Blesch, F Gage and M Tuszynski (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. Journal of Neuroscience 17(14): 5560-72.

Grimpe, B, Y Pressman, M Bunge and J Silver (2005) The role of proteoglycans in schwann cell/astrocyte interactions and in regeneration failure at pns/cns interfaces. Molecular and Cellular Neuroscience 28(1): 18-29.

Gundersen, R and J Barrett (1979) Neuronal chemotaxis: Chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206(4422): 1079-80.

Hannila, S and M Filbin (2008) The role of cyclic amp signaling in promoting axonal regeneration after spinal cord injury. Experimental Neurology 209(2): 321-32.

Himes, B, Y Liu, J Solowska, E Snyder, I Fischer and A Tessler (2001) Transplants of cells genetically modified to express neurotrophin-3 rescue axotomized clarke's nucleus neurons after spinal cord hemisection in adult rats. Journal of Neuroscience Research 65(6): 549-64.

Houle, J, V Tom, D Mayes, G Wagoner, N Phillips and J Silver (2006) Combining an autologous peripheral nervous system" bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. Journal of Neuroscience 26(28): 7405.

Houweling, D, A Lankhorst, W Gispen, P Bär and E Joosten (1998) Collagen containing neurotrophin-3 (nt-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. Experimental Neurology 153(1): 49-59.

Hudson, T, G Evans and C Schmidt (2000) Engineering strategies for peripheral nerve repair. Orthopedic Clinics of North America 31(3): 485-97.

Hurtado, A, L Moon, V Maquet, B Blits, R Jérôme and M Oudega (2006) Poly (d, l-lactic acid) macroporous guidance scaffolds seeded with schwann cells genetically modified to secrete a bi-functional neurotrophin implanted in the completely transected adult rat thoracic spinal cord. Biomaterials 27(3): 430-42.

Imperato-Kalmar, E, R Mckinney, L Schnell, B Rubin and M Schwab (1997) Local changes in vascular architecture following partial spinal cord lesion in the rat. Experimental Neurology 145(2): 322-28.

Jakeman, L, P Wei, Z Guan and B Stokes (1998) Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. Experimental Neurology 154(1): 170-84.

Jin, Y, I Fischer, A Tessler and J Houle (2002) Transplants of fibroblasts genetically modified to express bdnf promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Experimental Neurology 177(1): 265-75.

Jones, L, M Oudega, M Bunge and M Tuszynski (2001) Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. The Journal of Physiology 533(1): 83-89.

Jones, L, D Sajed and M Tuszynski (2003) Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: A balance of permissiveness and inhibition. Journal of Neuroscience 23(28): 9276-88.

Kelley, M and O Steward (1997) Injury-induced physiological events that may modulate gene expression in neurons and glia. Rev Neurosci 8(3-4): 147-77.

Kim, B, H Dai, J Lynskey, M Mcatee and B Bregman (2006) Degradation of chondroitin sulfate proteoglycans potentiates transplant-mediated axonal remodeling and functional recovery after spinal cord injury in adult rats. Journal of Comparative Biology 497(2): 182.

Kirik, D, C Rosenblad and A Bjorklund (2000) Preservation of a functional nigrostriatal dopamine pathway by gdnf in the intrastriatal 6-ohda lesion model depends on the site of administration of the trophic factor. European Journal of Neuroscience 12(11): 3871-82.

Kobayashi, N, D Fan, K Giehl, A Bedard, S Wiegand and W Tetzlaff (1997) Bdnf and nt-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate gap-43 and ta1-tubulin mrna expression, and promote axonal regeneration. Journal of Neuroscience 17(24): 9583-95.

Krenz, N and L Weaver (2000) Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. Journal of Neurochemistry 74(2): 730-39.

Langer, R and J Vacanti (1993) Tissue engineering. Science 260(5110): 920-26.

Letourneau, P (1978) Chemotactic response of nerve fiber elongation to nerve growth factor. Dev Biol 66(1): 183-96.

Liu, Y, D Kim, B Himes, S Chow, T Schallert, M Murray, A Tessler and I Fischer (1999) Transplants of fibroblasts genetically modified to express bdnf promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. Journal of Neuroscience 19(11): 4370-87.

Liu, Y, B Himes, J Solowska, J Moul, S Chow, K Park, A Tessler, M Murray, E Snyder and I Fischer (1999) Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. Experimental Neurology 158(1): 9-26.

Low, K, M Culbertson, F Bradke, M Tessier-Lavigne and M Tuszynski (2008) Netrin-1 is a novel myelin-associated inhibitor to axon growth. Journal of Neuroscience 28(5): 1099.

Lu, X and P Richardson (1991) Inflammation near the nerve cell body enhances axonal regeneration. Journal of Neuroscience 11(4): 972-78.

Lu, P, A Blesch and M Tuszynski (2001) Neurotrophism without neurotropism: Bdnf promotes survival but not growth of lesioned corticospinal neurons. injury 436: 456-70.

Lu, P, L Jones, E Snyder and M Tuszynski (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Experimental Neurology 181(2): 115-29.

Lu, P, H Yang, L Jones, M Filbin and M Tuszynski (2004) Combinatorial therapy with neurotrophins and camp promotes axonal regeneration beyond sites of spinal cord injury. Journal of Neuroscience 24(28): 6402-09.

Lu, P, L Jones and M Tuszynski (2005) Bdnf-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Experimental Neurology 191(2): 344-60.

Lu, P, L Jones and M Tuszynski (2007) Axon regeneration through scars and into sites of chronic spinal cord injury. Experimental Neurology 203(1): 8-21.

Lu, P and M Tuszynski (2008) Growth factors and combinatorial therapies for cns regeneration. Experimental Neurology 209(2): 313-20.

Ma, L, T Harada, C Harada, M Romero, J Hebert, S Mcconnell and L Parada (2002) Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. Neuron 36(4): 623-34.

Mason, M, A Lieberman, G Grenningloh and P Anderson (2002) Transcriptional upregulation of scg10 and cap-23 is correlated with regeneration of the axons

of peripheral and central neurons in vivo. Molecular and Cellular Neuroscience 20(4): 595-615.

Massey, J, J Amps, M Viapiano, R Matthews, M Wagoner, C Whitaker, W Alilain, A Yonkof, A Khalyfa and N Cooper (2008) Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase abc and neurotrophin-3. Experimental Neurology 209(2): 426-45.

Mckeon, R, R Schreiber, J Rudge and J Silver (1991) Reduction of neurite outgrowth in a model of glial scarring following cns injury is correlated with the expression of inhibitory molecules on reactive astrocytes. Journal of Neuroscience 11(11): 3398-411.

Menei, P, C Montero-Menei, S Whittemore, R Bunge and M Bunge (1998) Schwann cells genetically modified to secrete human bdnf promote enhanced axonal regrowth across transected adult rat spinal cord. European Journal of Neuroscience 10(2): 607-21.

Ming, G, A Lohof and J Zheng (1997) Acute morphogenic and chemotropic effects of neurotrophins on cultured embryonic xenopus spinal neurons. Journal of Neuroscience 17(20): 7860-71.

Moore, M, J Friedman, E Lewellyn, S Mantila, A Krych, S Ameenuddin, A Knight, L Lu, B Currier and R Spinner (2006) Multiple-channel scaffolds to promote spinal cord axon regeneration. Biomaterials 27(3): 419-29.

Mori, T, D Yamashita, J Ko-Ichi, K Shimizu and M Hayashi (2002) Changes in nt-3 and trkc in the primary visual cortex of developing macaques. NeuroReport 13(13): 1689.

Naldini, L, U Blomer, F Gage, D Trono and I Verma (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proceedings of the National Academy of Sciences 93(21): 11382-88.

Neumann, S and C Woolf (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23(1): 83-91.

Nikulina, E, J Tidwell, H Dai, B Bregman and M Filbin (2004) The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proceedings of the National Academy of Sciences 101(23): 8786-90.

Niwa, H, K Yamaura and J Miyazaki (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene(Amsterdam) 108(2): 193-99.

Palsson, B (2003). Tissue engineering, CRC Press.

Pearse, D, F Pereira, A Marcillo, M Bates, Y Berrocal, M Filbin and M Bunge (2004) Camp and schwann cells promote axonal growth and functional recovery after spinal cord injury. Nature Medicine 10: 610-16.

Pfeifer, A, M Ikawa, Y Dayn and I Verma (2002) Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proceedings of the National Academy of Sciences 99(4): 2140.

Pfeifer, K, M Vroemen, A Blesch and N Weidner (2004) Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury. European Journal of Neuroscience 20(7): 1695-704.

Pizzi, M and M Crowe (2006) Transplantation of fibroblasts that overexpress matrix metalloproteinase-3 into the site of spinal cord injury in rats. Journal of Neurotrauma 23(12): 1750-65.

Plant, G, C Christensen, M Oudega and M Bunge (2003) Delayed transplantation of olfactory ensheathing glia promotes sparing/regeneration of supraspinal axons in the contused adult rat spinal cord. Journal of Neurotrauma 20(1): 1-16.

Plunet, W, B Kwon and W Tetzlaff (2002) Mini-review promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. Journal of Neuroscience Research 68: 1-6.

Popovich, P, P Wei and B Stokes (1997) Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats. The Journal of Comparative Neurology 377(3): 443-64.

Pruss, R (1979) Thy-1 antigen on astrocytes in long-term cultures of rat central nervous system. Nature 280(5724): 688-90.

Prussin, C and D Metcalfe (2003) 4. Ige, mast cells, basophils, and eosinophils. The Journal of Allergy and Clinical Immunology 111(2S2): 486-94.

Qiu, J, D Cai, H Dai, M Mcatee, P Hoffman, B Bregman and M Filbin (2002) Spinal axon regeneration induced by elevation of cyclic amp. Neuron 34(6): 895-903.

Ramer, M, J Priestly and S Mcmahon (2000) Functional regeneration of sensory axons into the adult spinal cord. Nature(London) 403(6767): 312-16.

Ratner, B (1997). Biomaterials science: An introduction to materials in medicine, Academic Press.

Reich, J, D Burmeister, J Schmidt and B Grafstein (1990) Effect of conditioning lesions on regeneration of goldfish optic axons: Time course of the cell body reaction to axotomy. Brain research 515(1-2): 256-60.

Richardson, P and V Issa (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309(5971): 791-93.

Ringstedt, T (1997) Limb proprioceptive deficits without neuronal loss in transgenic mice overexpressing neurotrophin-3 in the developing nervous system. Development 124(13): 2603-13.

Romero, M, N Rangappa, M Garry and G Smith (2001) Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. Journal of Neuroscience 21(21): 8408.

Ross, R (1968) The fibroblast and wound repair. Biological Reviews 43(1): 51-91.

Ruitenberg, M, G Plant, F Hamers, J Wortel, B Blits, P Dijkhuizen, W Gispen, G Boer and J Verhaagen (2003) Ex vivo adenoviral vector-mediated neurotrophin gene transfer to olfactory ensheathing glia: Effects on rubrospinal tract regeneration, lesion size, and functional recovery after implantation in the injured rat spinal cord. Journal of Neuroscience 23(18): 7045-58.

Sabine Conrad, T, H Schluesener, M Adibzahdeh and J Schwab (2005) Spinal cord injury induction of lesional expression of profibrotic and angiogenic connective tissue growth factor confined to reactive astrocytes, invading fibroblasts and endothelial cells. J. Neurosurg Spine 2: 319-26.

Schmidt, C and J Leach (2003) N eural t issue e ngineering: Strategies for repair and regeneration. Annual Reviews in Biomedical Engineering 5(1): 293-347.

Schnell, L, R Schneider, R Kolbeck, Y Barde and M Schwab (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367(6459): 170-73.

Schwab, M, J Kapfhammer and C Bandtlow (1993) Inhibitors of neurite growth. Annual Reviews in Neuroscience 16(1): 565-95.

Shichinohe, H, S Kuroda, S Tsuji, S Yamaguchi, S Yano, J Lee, H Kobayashi, S Kikuchi, K Hida and Y Iwasaki (2008) Bone marrow stromal cells promote neurite extension in organotypic spinal cord slice: Significance for cell transplantation therapy. Neurorehabilitation and Neural Repair 22(5): 447.

Silver, J and J Miller (2004) Regeneration beyond the glial scar. Nature reviews. Neuroscience(Print) 5(2): 146-56.

Snider, W, F Zhou, J Zhong and A Markus (2002) Signaling the pathway to regeneration. Neuron 35(1): 13-16.

Spencer, T and M Filbin (2004) A role for camp in regeneration of the adult mammalian cns. Journal of Anatomy 204(1): 49-55.

Steward, O, B Zheng and M Tessier-Lavigne (2003) False resurrections: Distinguishing regenerated from spared axons in the injured central nervous system. spinal cord 459: 1-8.

Steward, O, B Zheng, M Tessier-Lavigne, M Hofstadter, K Sharp and K Yee (2008) Regenerative growth of corticospinal tract axons via the ventral column after spinal cord injury in mice. Journal of Neuroscience 28(27): 6836.

Stokols, S and M Tuszynski (2004) The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. Biomaterials 25(27): 5839-46.

Stokols, S and M Tuszynski (2006) Freeze-dried agarose scaffolds with uniaxial channels stimulate and guide linear axonal growth following spinal cord injury. Biomaterials 27(3): 443-51.

Stokols, S, J Sakamoto, C Breckon, T Holt, J Weiss and M Tuszynski (2006) Templated agarose scaffolds support linear axonal regeneration. Tissue Engineering 12(10): 2777-87.

Taylor, L, L Jones, M Tuszynski and A Blesch (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. Journal of Neuroscience 26(38): 9713.

Tessarollo, L, V Coppola and B Fritzsch (2004) Nt-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea. Journal of Neuroscience 24(10): 2575-84.

Tessier-Lavigne, M (1994) Axon guidance by diffusible repellants and attractants. Curr Opin Genet Dev 4(4): 596-601.

Tetzlaff, W, N Kobayashi, K Giehl, B Tsui, S Cassar and A Bedard (1994) Response of rubrospinal and corticospinal neurons to injury and neurotrophins. Prog Brain Res 103: 271-86.

Tokumine, J, O Kakinohana, D Cizkova, D Smith and M Marsala (2003) Changes in spinal gdnf, bdnf, and nt-3 expression after transient spinal cord ischemia in the rat. Journal of Neuroscience Research 74(4): 552-61.

Tucker, K, M Meyer and Y Barde (2001) Neurotrophins are required for nerve growth during development. Nature Neuroscience 4: 29-37.

Tuszynski, M, K Gabriel, F Gage, S Suhr, S Meyer and A Rosetti (1996) Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. Experimental Neurology 137(1): 157-73.

Tuszynski, M, J Conner, A Blesch, D Smith, D Merrill and H Vahlsing (2002) New strategies in neural repair. Plasticity in the Adult Brain: From Genes to Neurotherapy.

Tuszynski, M, R Grill, L Jones, H Mckay and A Blesch (2002) Spontaneous and augmented growth of axons in the primate spinal cord: Effects of local injury and nerve growth factor-secreting cell grafts. The Journal of Comparative Neurology 449(1): 88-101.

Weidner, N, A Blesch, R Grill and M Tuszynski (1999) Nerve growth factor–hypersecreting schwann cell grafts augment and guide spinal cord axonal growth and remyelinate central nervous system axons in a phenotypically appropriate manner. The Journal of Comparative Neurology 413: 495-506.

Wong, D, J Leveque, H Brumblay, P Krebsbach, S Hollister and F Lamarca (2008) Macro-architectures in spinal cord scaffold implants influence regeneration. Journal of Neurotrauma 25(8): 1027-37.

Woolf, C (2001) Turbocharging neurons for growth: Accelerating regeneration in the adult cns. Nature Neuroscience 4: 7-9.

Xu, X, V Guénard, N Kleitman, P Aebischer and M Bunge (1995) A combination of bdnf and nt-3 promotes supraspinal axonal regeneration into schwann cell grafts in adult rat thoracic spinal cord. Experimental Neurology 134(2): 261-72.

Y Cajal, R S.(1928) degeneration and regeneration of the nervous system. Hafner, New York.

Yang, H, P Lu, H Mckay, T Bernot, H Keirstead, O Steward, F Gage, V Edgerton and M Tuszynski (2006) Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. Journal of Neuroscience 26(8): 2157-66.

Ye, J and J Houle (1997) Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Experimental Neurology 143(1): 70-81.

Yick, L, P Cheung, K So and W Wu (2003) Axonal regeneration of clarke's neurons beyond the spinal cord injury scar after treatment with chondroitinase abc. Experimental Neurology 182(1): 160-68.

Yin, Y, Q Cui, Y Li, N Irwin, D Fischer, A Harvey and L Benowitz (2003) Macrophage-derived factors stimulate optic nerve regeneration. Journal of Neuroscience 23(6): 2284.

Yin, Y, M Henzl, B Lorber, T Nakazawa, T Thomas, F Jiang, R Langer and L Benowitz (2006) Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. Nature Neuroscience 9: 843-52.

Zhang, Y, P Dijkhuizen, P Anderson, A Lieberman and J Verhaagen (1998) Nt-3 delivered by an adenoviral vector induces injured dorsal root axons to regenerate into the spinal cord of adult rats. J Neurosci Res 54(4): 554-62.

Zhou, L, B Baumgartner, S Hill-Felberg, L Mcgowen and H Shine (2003) Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. Journal of Neuroscience 23(4): 1424.

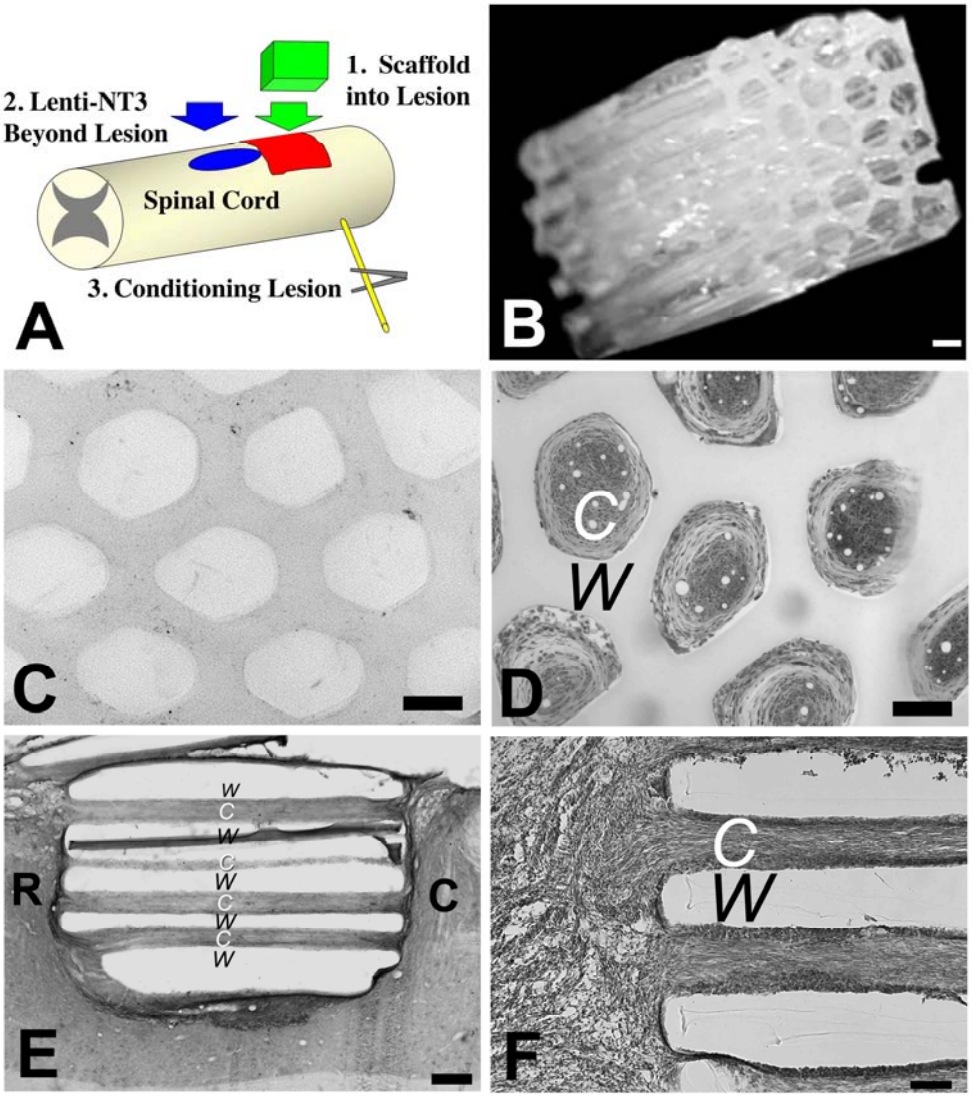

Fig. 2.1 Macro- and micro-scopic structures of agarose scaffolds. A) Subjects underwent dorsal column lesions at C4, scaffold implantation, injection of lentiviral vectors expressing NT-3 rostral to the lesion, and conditioning lesions of the sciatic nerve. B) Gross scaffold architecture. C) Cross-section of scaffold channels, demonstrating scaffold architecture and channel diameter of 200um. D) Toluidene Blue stain of scaffold 4 wk after implantation into dorsal column lesion site (coronal section). *W*, walls of scaffold; *C*, channels of scaffold (containing cells). E, F) Nissl stain in sagittal plane demonstrates scaffold integration into lesion site; *W*, walls of scaffold; *C*, channels of scaffold (containing cells). **R** and **C** label rostral and caudal aspects of lesion, respectively. Scale bars: B) 200um, C) 100um, D) 250um, E),F) 100um.

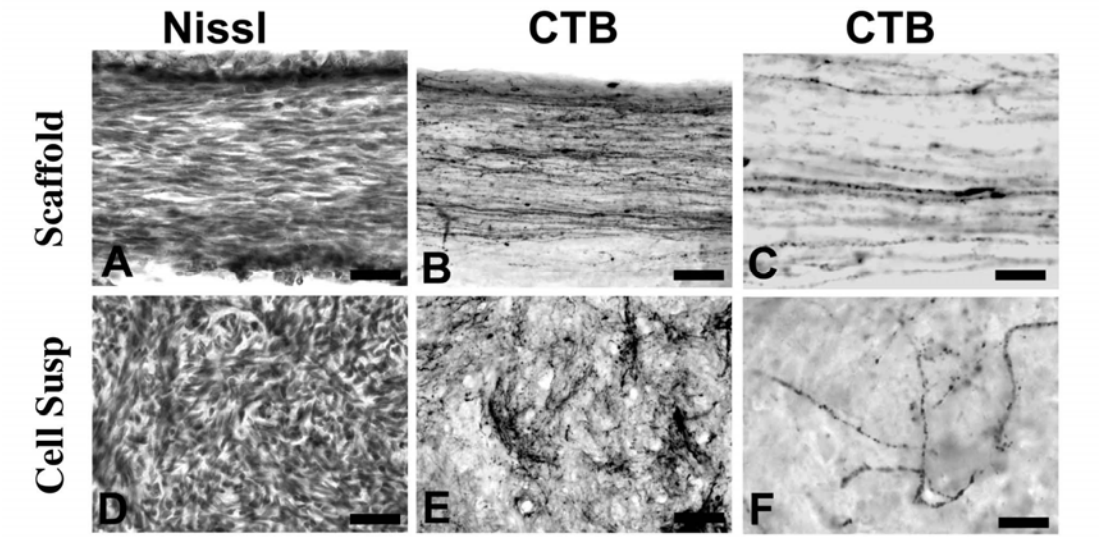

Fig. 2.2 Cellular and axonal orientation within scaffolds. A) 4 wk after implantation into spinal cord lesion site, cells within scaffolds are oriented in a linear manner corresponding to the rostral-caudal orientation of the spinal cord (Nissl stain). B, C) CTB-labeled sensory axons regenerating into scaffold also exhibit a highly linear organization corresponding to the rostral-caudal orientation of the spinal cord. D-F) In contrast, random orientation of cells (D, Nissl) and axons (CTB label, E, F) is observed in subjects that received non-organized, cell suspension grafts into lesion cavity, without scaffold. Scale bars A, B, D, E, 50μm; C, F, 25μm.

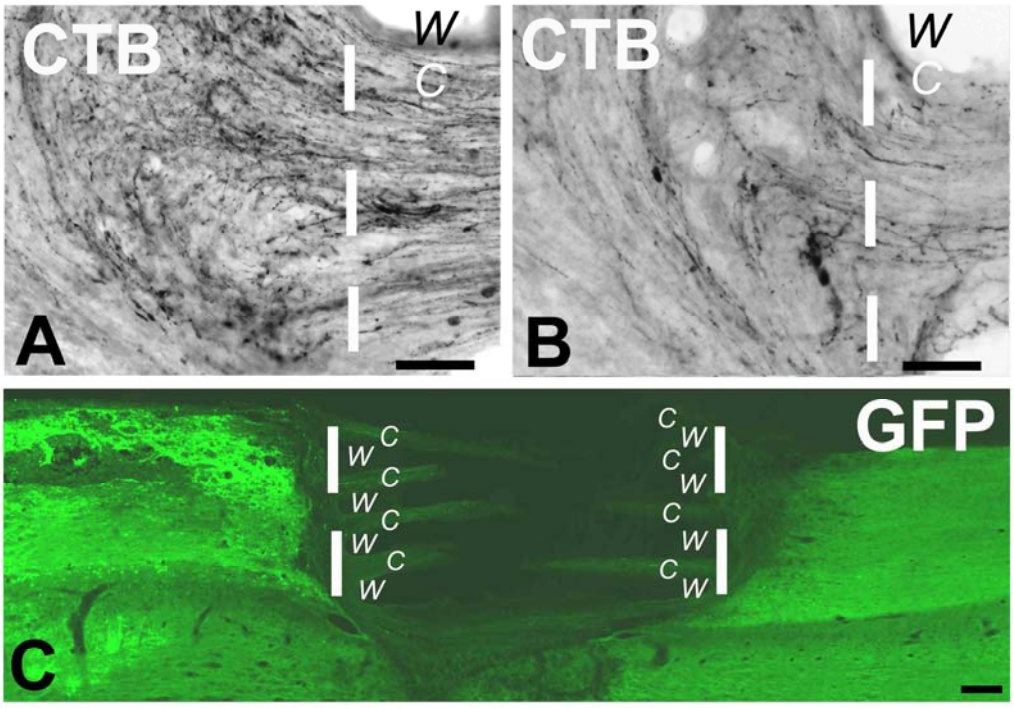

Fig. 2.3 Axon emergence from scaffolds. A) Numerous CTB-labeled sensory axons exit scaffold in rostral aspect of lesion site when subjected to combinatorial therapy with lentiviral NT-3 injection rostral to the lesion site, and conditioning lesions of sciatic nerve. Vertical lines indicate scaffold/lesion interface at rostral aspect of lesion cavity; scaffold to right, axon emergence to left. B) Fewer CTB-labeled sensory axons emerge from scaffold when lenti-NT-3 is not injected rostral to lesion site; this subject did receive a conditioning lesion. C) GFP immunolabel indicates zone of NT-3 transduction rostral to lesion site; providing chemotropic NT-3 gradient to attract emergence of axons from scaffold. *W*, walls of scaffold; *C*, channels of scaffold (containing cells); vertical lines indicate end of scaffold. Scale bar A, B, 50μm; C, 250μm.

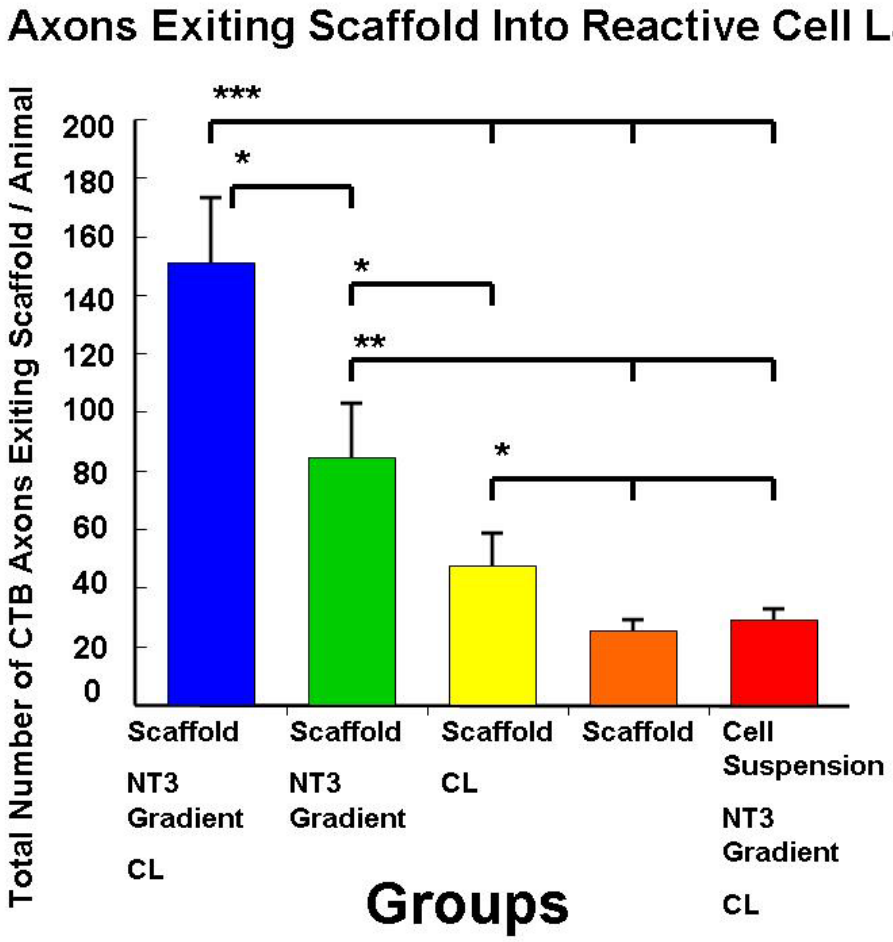

Fig. 2.4 Quantification of axons exiting scaffolds. The number of axons per subject exiting scaffolds was quantified in each group as described in methods. Axons exit scaffold to penetrate the reactive cell layer between scaffold implant and host spinal cord. Labels identify treatment in each group; "scaffold" group received injection of NT-3-secreting MSCs into scaffold prior to in vivo implantation; "Empty scaffold" group received an empty scaffold in lesion site. Subjects that received full combinatorial therapy exhibitied significantly greater axons reaching the end of scaffolds compared to all other groups. Of note, subjects that received cell suspension grafts in the lesion cavity, plus full combination therapy, showed substantially fewer axons reaching distal end of scaffold compared to combination-treated, scaffold-implanted subjects. Overall group differences were present by ANOVA ($P<0.01$); post-hoc differences are shown with asterisks. * $p< 0.05$; ** $p<0.01$; *** $p<0.001$).

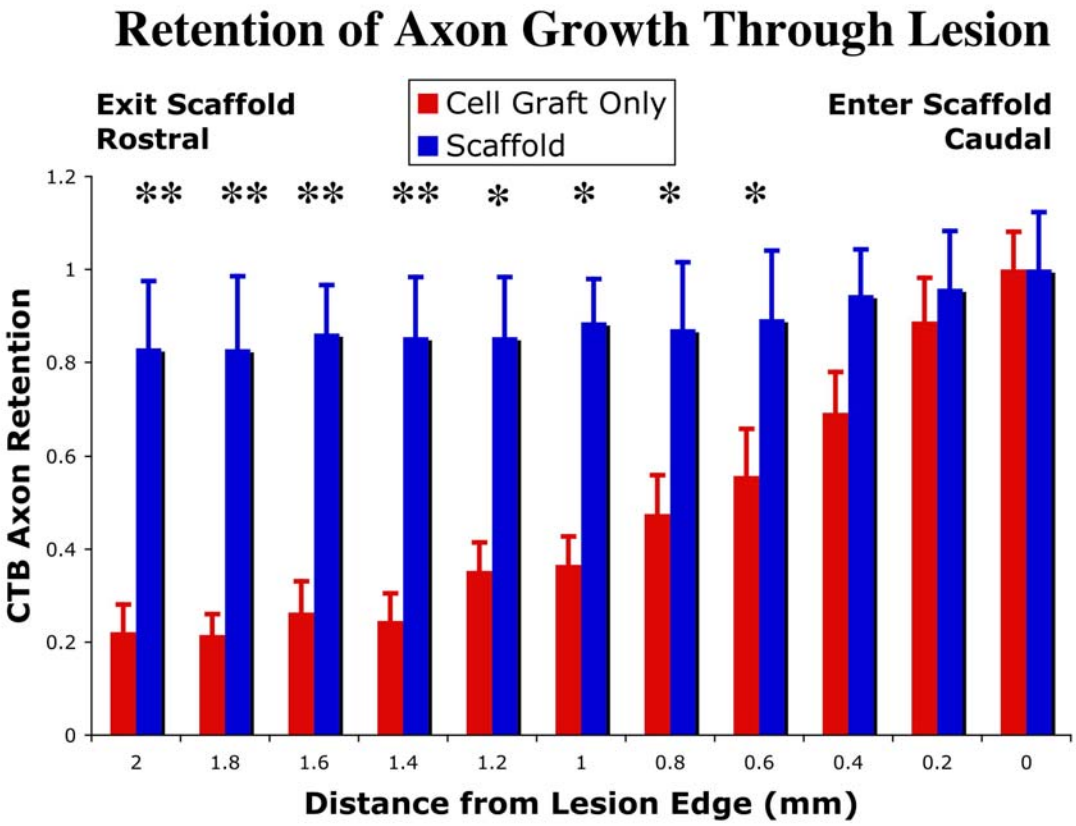

Fig. 2.5 Axon number is preserved over distance in scaffold-implanted subjects. Quantification of CTB-labeled sensory axon number at 200µm intervals through scaffold or cell suspension graft. Note that sensory axons penetrate scaffold from caudal aspect of lesion (right side of graph) and emerge from rostral aspect of lesion (left side of graph). Sensory axon numbers are retained over extended distances through the lesion site only in scaffold-implanted subjects. Subjects with non-organized, cell suspension grafts exhibit rapid degradation of axon number as distance from entry to lesion site increases. Both groups received full combination treatment. ANOVA $p<0.05$; comparisons between groups at each distance shown as t-test (* $p<0.05$, ** $p<0.01$).

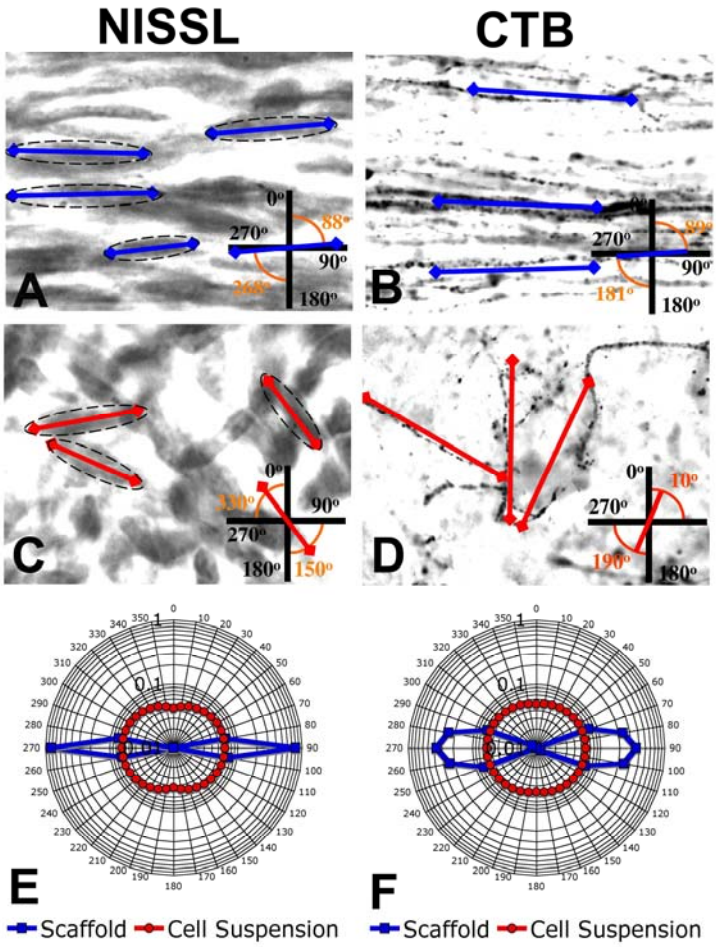

Fig. 2.6 Cellular and axon linearity are increased within scaffold channels. A) Nissl stained cells were approximated as ellipses and the angle of the two ends of the cell was measured with respect to the longitudinal orientation of the scaffold or host spinal cord. B) CTB labeled axons were averaged as linear vectors every 100um and the angle was measured with respect to the dorsal aspect. Each animal was randomly sampled three times in a 200um square within the lesion. C) Nissl orientation within the scaffold is highly linear compared to a cell graft. D) CTB orientation within the scaffold is highly linear compared to the cell suspension-grafted animals. E) Using a general cell marker for Nissl substance (thionin stain), 80 $\pm$ 5% of cells within scaffolds were linearly arranged along the rostral-to-caudal axis of the spinal cord, whereas only 6 $\pm$ 5% of sampled cells were oriented in the rostral-to-caudal axis of the spinal cord in subjects that received randomly organized cell suspension grafts. F) Using a transganglionic axonal tracer, CTB, 36 + 5% of axons were linearly arranged along the rostral-to-caudal axis of the spinal cord, whereas 6 + 5% of sampled axons were oriented in the rostral-to-caudal axis of the spinal cord in subjects that received randomly organized cell suspension grafts.

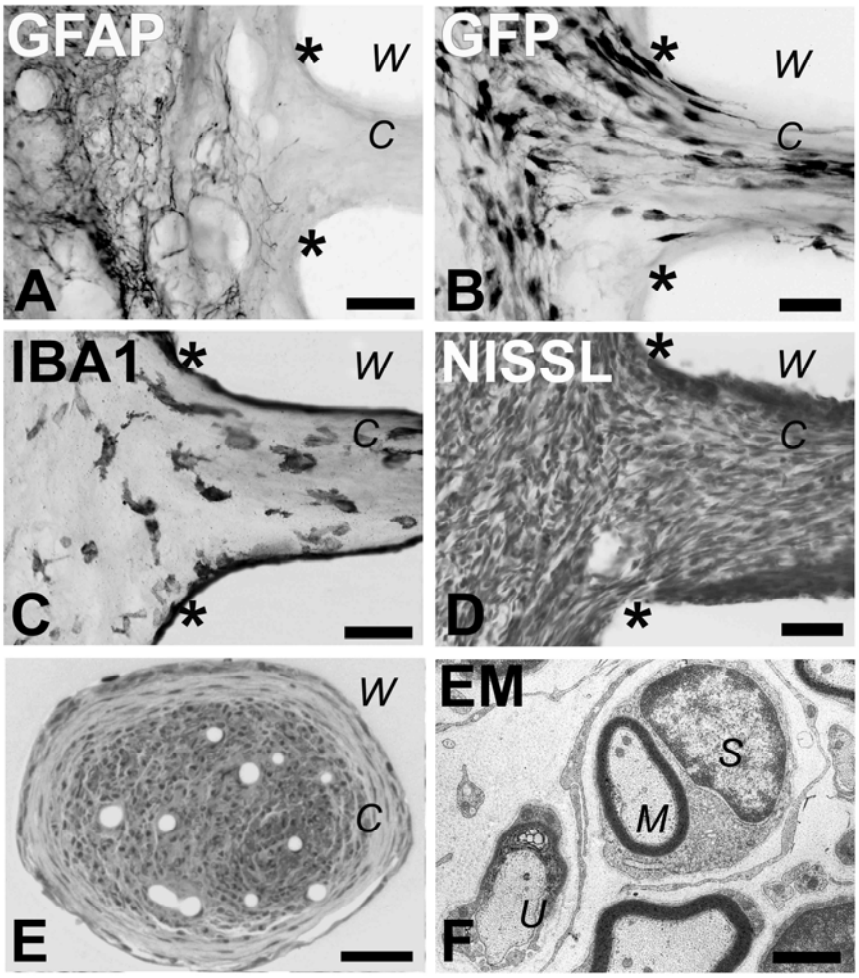

Fig. 2.7 Cellular markers at rostral scaffold interface. A) GFAP immunoreactivity does not penetrate scaffold channels. Asterisks in all panels show rostral termination of scaffold, indicating rostral scaffold/host interface. B) GFP labeled cells expressing NT-3 are present within host spinal cord rostral to the lesion site, and extend into scaffold channels. Thus, lent-NT3 injections diffuse from the injection site 2.5mm rostral to the lesion, and reach the scaffold itself. C) IBA1 label indicates a moderate microglial response at rostral scaffold interface. D) Nissl stain shows dense cell numbers within scaffolds with minimal granularized tissue at the scaffold interface. E) Toluidine blue stain in semithin section shows cell accumulation within scaffold channel (located 200µm from rostral end of scaffold), and numerous blood vessels. *W*, wall of scaffold; *C*, channel of scaffold. F) Electron microscopy demonstrates myelinated (*M*) and unmyelinated (*U*) axons within scaffold channels, and Schwann cells (*S*) generating myelin. Oligodendrocytes were never observed within scaffolds. Scale bars A, B, 100µm; C, D, 50µm; E, 40µm; F, 3µm.

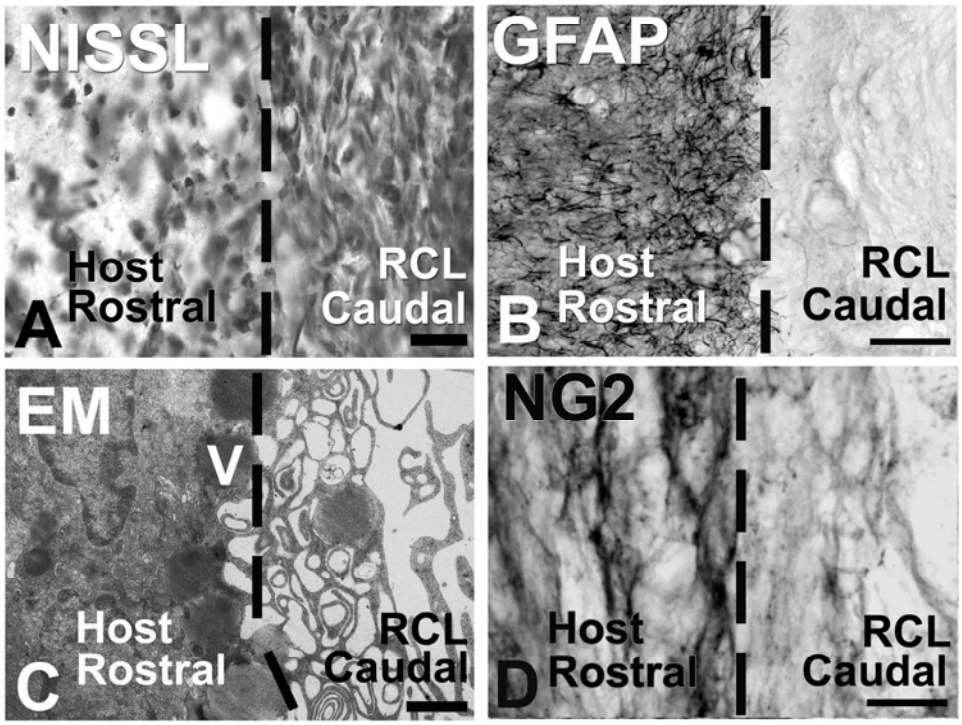

Fig. 2.8 Reactive cell layer adjacent to scaffold implant. CTB-labeled sensory axons emerge from rostral ends of scaffolds in substantial numbers when animals receive combinatorial therapies, as indicated in preceding figures, but axons do not re-penetrate spinal cord gray or white matter beyond scaffold. Instead, axons emerging from scaffolds are confined to a reactive cell layer interposed between scaffold and host tissue, shown in this figure. Sagittal sections. A) Nissl stain shows a clear distinction in cellular morphology between reactive cell layer (RCL) and host spinal cord immediately surrounding the lesion site. Cells within reactive cell layer exhibit fine, linear orientation suggestive of fibroblasts and leptomeningeal cells. B) GFAP immunreactivity indicates a sharp demarcation between host spinal cord and the reactive cell layer; regenerating axons did not regenerate beyond this glial boundary. C) At the ultrastructural level, the fibroblastic and leptomeningeal identity of cells within reactive cell layer apposed to scaffold are confirmed. Large, electron-dense vacuolar (*V*) accumulations are also observed both within reactive cell layer at the interface with host tissue. D) Labeling for the inhibitory chondroitin sulfate proteoglycan molecule NG2 also indicates that inhibitory matrix defines the boundary between reactive cell layer and the host spinal cord beyond. Thus, glial and extracellular matrix molecules may constitute a barrier preventing re-penetration of axons from scaffolds into host tissue. Scale bars A, 25µm; B, 50µm; C, 5µm; D, 50µm.

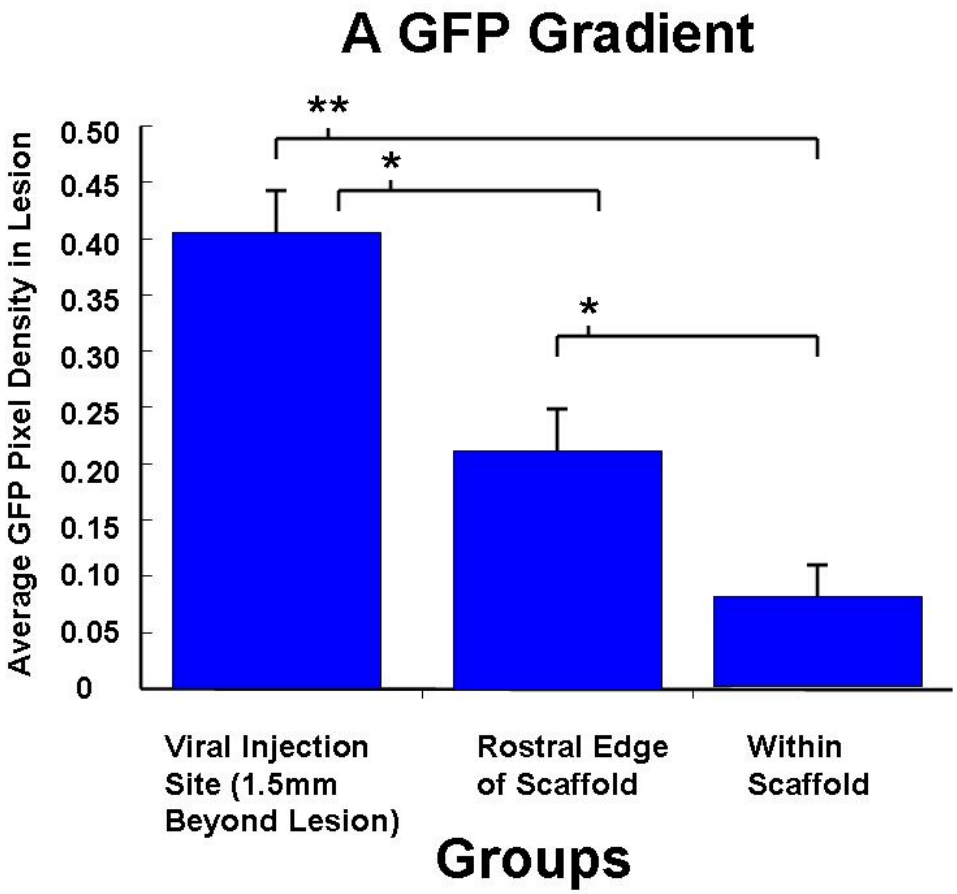

Fig. 2.S1 A gradient of GFP immunoreactivity was created by lentiviral-GFP gene injection 1.5mm rostral to the lesion site. The pixel density of GFP-transduced cells within a 200µm square region located 1.5mm beyond the lesion site, the rostral aspect of the scaffold, and within the scaffold was compared. The highest density of GFP cells was present beyond the lesion site at the site of viral injection. Furthermore, the rostral aspect of the lesion had a higher GFP pixel density than within the scaffold, suggesting that a neurotrophic gradient was created that extended beyond the lesion site and into the region of axonal egress from scaffolds. ANOVA $p<0.05$; post hoc Fisher's t-test * $p<0.05$, ** $p<0.01$.

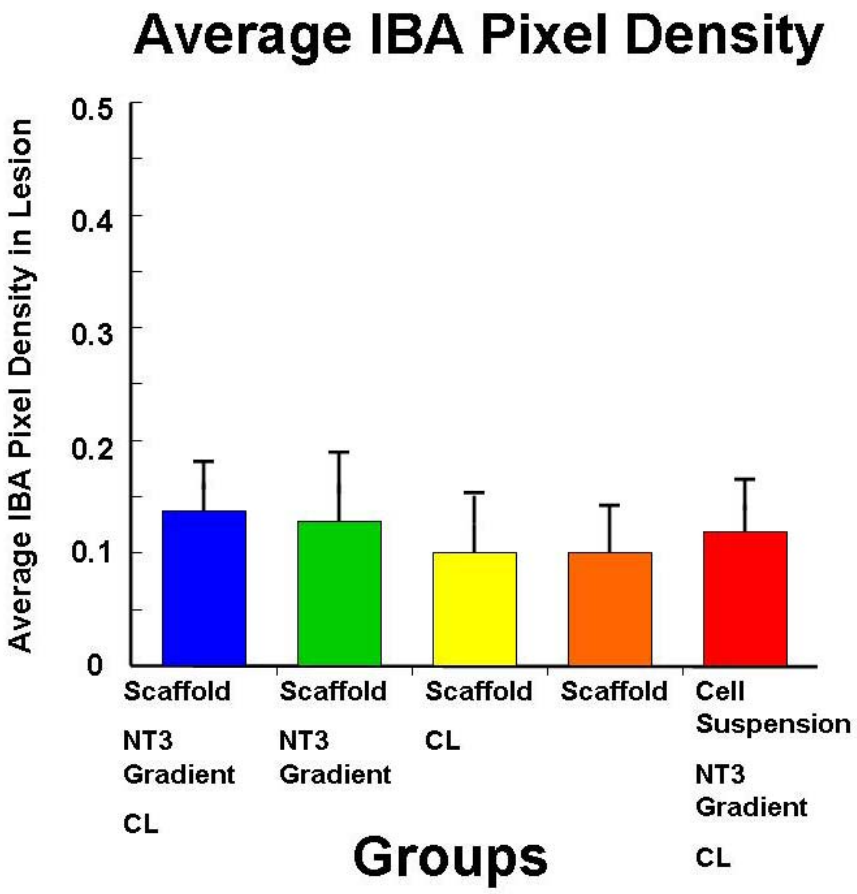

Fig. 2.S2 Microglial response to scaffold implantation. The proportion of a 200µm x 200µm field occupied by IBA-1 reaction product (a marker of microglial cells) within the rostral region of the scaffold channels (or cell suspension lesion) did not differ significantly between groups that received scaffold implants versus cell suspension grafts. Six samples for each animal were randomly sampled within the rostral aspect of the scaffold channels (or cell suspension lesion) for quantification. The center and caudal regions of the scaffold (and cell suspension) were also quantified and showed no statistical differences (data not shown).

CHAPTER 3

Templated agarose scaffolds orient and guide regenerating axons after complete spinal cord transection

Abstract

Previously we reported that a combinatorial therapy using a templated agarose scaffold, a neutrotrophic source, and a conditioning lesion can linearly orient cellular deposition within a lesion and guide *ascending* sensory axons across a dorsal column injury. In the present study we examined whether growth of *descending* axons could be linearly oriented and guided across a complete transection by delivering a chemo-attractant protein (BDNF) and a templated agarose scaffold. Adult rats underwent complete transection of the spinal cord at the T10 level and six animals received a templated agarose scaffold seeded with autologous stromal cells genetically modified to secrete BDNF into the lesion. In addition, subjects received injections of lentiviral vectors expressing BDNF beyond the lesion site, because previous studies have indicated that only combinatorial treatments support bridging axonal regeneration after spinal cord injury (Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004). Two control groups were studied: animals with complete transections that received the same combination therapy, but received non-organized cell suspension grafts into the lesion cavity rather than cells injected into templated scaffolds; and subjects that underwent complete transections without further treatment. Sixteen weeks after spinal

cord transection, lesion-only controls exhibited empty cystic cavities that were devoid of axons. In contrast, subjects that received combinatorial therapies without scaffolds exhibited a cellular fill within the lesion site that supported axonal extension. Notably, subjects that received combinatorial therapies and templated agarose scaffolds exhibited a remarkable, linear organization to descending regenerating axons. Constraint of patterns of axonal regeneration to linear arrays within the 200μm cross-sectional diameter of scaffold channels resulted in the retention of 85±14% of axons across the full length of the lesion cavity, compared to 0% retention in combinatorially-treated subjects that received non-organized, cell suspension grafts into the lesion cavity. However, formation of a reactive cell layer at the distal scaffold-host interface prevented axon re-penetration of spinal cord beyond the scaffold. Thus, templated agarose scaffolds exhibit the capability to impart microstructural guidance to regenerating axons, significantly enhancing the extent of axonal regeneration after complete spinal transection, but reactive cellular interfaces must be reduced to support host reinnervation.

Introduction

Severe spinal cord contusion results in cystic degeneration at the lesion site, leading to large spinal cavities that are non-conducive to axon growth (Tator et al., 1991; Tator et al.,1995; Quencer et al., 1996). Often a lesion cavity can be filled with a cellular substrate to provide a permissive substrate for axon growth (Ronsysn et al., 2008). The resulting cellular orientation within the lesion is typically random, creating

a spatially disorganized environment for axon growth. This cellular disorganization has been addressed in previous studies by using innate linear architecture within peripheral nerve grafts (Cheng et al., 1996; Oudega et al., 1994; David et al., 1981).

Furthermore, injured axons regenerate more extensively when additional neurotrophic support is supplied to an injury site (Tuszynski et al., 1996; Bradbury et al., 1999; Liu et al., 1999a). Several studies indicate that neurotrophins and other diffusible proteins may provide guidance cues for both developing and adult, regenerating axons (Letourneau et al., 1978; Gundersen and Barrett, 1979; Gallo et al., 1997; Paves and Saarma, 1997; Ringstedt et al., 1997; Houweling et al., 1998; Zhang et al., 1998; Kirik et al., 2000; Romero et al., 2001; Tucker et al., 2001; Dontchev and Letourneau, 2002; Zhou et al., 2003; Genc et al., 2004; Tessarollo et al., 2004). BDNF in particular is a nervous system growth factor that has been shown to promote the growth of various descending motor axons (Oudega and Hagg, 1999; Liu et al., 1999b). Additional data indicates that transected descending axons can penetrate cell suspension grafts expressing brain derived neurotrophic factor (BDNF) in sites of spinal cord injury (Lu et al., 2005).

We have previously engineered a biocompatible hydrogel with highly linear 200-micron sized channels which supports *ascending* axon regeneration across a large spinal cord injury (Stokols et al., 2006). However, functional recovery is only possible through the regeneration of *descending* motor axons. Thus, here we test the hypothesis that by constraining regenerating axons into a linear array we may enhance the number of *descending* axons crossing a large complete transection (2mm). We

used a linearized agarose scaffold to constrain axonal growth in a linear manner within the lesion. Due to recent successes of multi-component approaches to promote axon growth beyond a lesion site, we applied a combination of a cell suspension genetically modified to secrete BDNF within the lesion cavity and a lenti-viral mediated BDNF growth factor gradient beyond the site of injury to enhance descending axon growth across the complete transection and into the host parenchyma. To examine the potential of the linearized scaffold to enhance the number of *descending* axons crossing a large lesion, two groups received a gene therapy beyond the lesion site, of lenti-BDNF, while in the lesion cavity, one group received a linearized scaffold seeded with genetically modified cells and the other group received a disorganized cell suspension of genetically modified cells (BMSC-BDNF). Furthermore, both groups were compared to a lesion only control group.

Materials and methods

Experimental design

Three groups of animals were studied. All groups underwent T10 spinal cord complete transections, as described below. Group 1 (n=6) received implants of templated agarose scaffolds into the lesion cavity. Scaffolds were pre-seeded with autologous marrow stromal cells that secreted BDNF by careful dehydration with a UV sterilized Kim wipe and immediate rehydration with the cell suspension at $1x10^6$ cells/ml. In addition to scaffold implants, these animals received injections of

lentiviral vectors expressing BDNF 1.5 mm beyond (caudal to) the T10 lesion site to elicit axonal egress from scaffolds. Group 2 (n=6) received full combinatorial therapy as in Group 1, but instead of receiving implants of templated scaffolds, this group received cell suspension grafts of BDNF-secreting cells into the lesion cavity (a non-organized cellular matrix). Group 3 (n=6), a control, underwent a T10 spinal cord complete transection with no treatments. Three days before perfusion, animals received injections of the anterograde tracer biotinylated dextran amine (BDA) 2mm rostral to lesion site to trace the corticospinal and rubrospinal descending tracts. Subjects survived 4 months after lesions and treatments.

Fabrication and sterilization of scaffolds

Multi-component fiber bundles (MCFBs) were used to generate precise linear channels, as previously described (Stokols 2006). Briefly, to generate scaffolds with precise linear channels, multi-component fiber bundles (MCFBs) were manufactured from 200-mm-diameter polystyrene fibers (Paradigm Optics, Vancouver, WA). Fibers were arranged in a hexagonal close-packed array separated by a continuous matrix of poly(methyl methacrylate) (PMMA). MCFBs were simultaneously extruded and fused such that the polystyrene fibers were oriented parallel to the longitudinal axis of the bundles. The MCFBs were trimmed to 2 mm longitudinal length and cross-sectional diameter of 3 mm. Polystyrene end caps of 4 mm length were bonded to the ends of the MCFBs using a 1:1 acetone/toluene (v/v) mixture to anchor polystyrene fibers within the MCFB to an external, rigid material. Next, polystyrene side caps were

bonded to the ends of MCFBs. The PMMA matrix was then selectively removed by immersing MCFB templates in 99.7% propylene carbonate (Sigma-Aldrich) at 45^{o}C for 24 hr; this was repeated 3 times to ensure complete removal of PMMA. The templates were then washed in 95% ethanol and distilled water prior to agarose casting.

Ultrapure agarose (30 mg/mL; Sigma-Aldrich) was dissolved in distilled water at 100^{o}C. After cooling of agarose to 65^{o}C, MCFB templates were submerged into the agarose solution and centrifuged at 300rpm for 20 sec to overcome surface tension between the agarose and closely packed polystyrene fibers. After maintenance at 65^{o}C for 5 min, the heat source was removed and agarose was allowed to gel at room temperature. MCFB templates, now permeated with gelled agarose, were cut from the gelled agarose aggregate, leaving just an agarose scaffold cast, bound by polystyrene caps and fiber mold. The polystyrene fibers, end-caps, and side-caps were dissolved by immersing templates in 99% tetrahydrofuran (THF) (Sigma-Aldrich) at room temperature for 24 hr. This process was repeated 3 times to ensure complete removal of polystyrene, resulting in individual free-floating agarose scaffolds. Scaffolds were collected and washed in acetone, then stored in 95% ethanol for sterile storage until future use. One day before implantation the scaffolds were washed in 3 cycles of autoclaved distilled water and stored in a sterile UV hood until implantation. Additionally, to create an elliptical shape mimicking the spinal cord, the scaffolds were briefly frozen and cut with a thin, flexible sheet metal formed into the shape of the complete transection.

Analysis of scaffold architecture

Scaffold microstructure was visualized under phase-contrast microscopic optics, as previously described (Stokols et al., 2006). Pore dimensions and porosity were directly measured from light micrographs. Templated agarose scaffolds with channel diameters of 200μm and a uniform wall thickness of 100μm were consistently produced. Scaffolds contained uniaxial channels organized in an array precisely replicating the pattern of polystyrene fiber cores in the MCFB. Scaffold microstructure was also examined under light level microscopy, and the rare scaffold with imperfections of channel architecture was not used for *in vivo* studies.

Cell culture

Rat primary marrow stromal cells (MSCs) were isolated according to the method of Azizi et al., 1998. Briefly, Fischer 344 adult female rats were anesthetized with a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 g/ml), and acepromazine (0.25 mg/ml), and tibias and femurs were dissected free. After sectioning free the end of each bone, 5 ml of MSC culture medium consisted of alpha-MEM (Gibco/BRL) supplemented with 20% fetal bovine serum (FBS) and antibiotics was injected into the central canal of the bone to extrude the marrow. Whole marrow cells were extracted and cultured at a density of 5–10 x 10^5 cells/cm^2 in MSC culture medium. The nonadherent cells were removed after 24 h by change of medium and the medium was changed every other day until cells became confluent. The cells were

split 1:3 and passaged three times. The cells were then frozen in aliquots and stored in liquid nitrogen.

Transduction of MSCs with the GFP reporter gene

To track grafted cells in vivo, MSCs injected were transduced with the jellyfish green fluorescent protein (GFP) reporter gene as described previously (Lu et al., 2003). Briefly, the retroviral vector plasmid DNA pLXSN-GFP was transfected into the stable viral producer line PA317 utilizing the ecotropic packaging cell line psi-2 as described previously (Blesch et al., 2004 and Tuszynski et al., 1996). The virus collected from 24 h conditioned medium was used to infect primary MSCs. GFP-expressing MSCs (MSC-GFP) were analyzed and sorted following standard procedures by a FACS scan directed to detection of GFP fluorescence, with a standard excitation wavelength of 488 nm.

Transduction of MSCs with the BDNF gene

MSCs grafted into some groups of subjects were transduced to overexpress both human BDNF and GFP (as a reporter gene). The production of pLXIE-BDNF retrovirus was previously described in (Lu et al., 2005). Briefly, the latter plasmid is a retroviral vector containing multiple cloning sites (MCS) and an internal ribosome entry site (IRES) that allows read-through expression in the same cells of enhanced GFP (Lu et al., 2003). The plasmid pLXIE-BDNF was transfected into the Phoenix producer cell line (Kinsella and Nolan, 1996), and BDNF-transduced MSCs (“MSC-

BDNF-GFP") were fluorescently sorted by FACS directed to detection of GFP fluorescence.

Production of lentiviral vectors

The vector pLV (Pfeifer et al., 2002) was used to construct BDNF-secreting and control green fluorescent protein (GFP)-expressing vectors. The GFP and BDNF lentiviral vector have been described previously in Kwon, 2007 (Kwon et al., 2007). Briefly, self-inactivating lentiviral vectors were produced and concentrated using an ultracentrifuge. The lentiviral vector expressed the human BDNF cDNA and also enhanced GFP using an internal ribosome entry site under control of the *B*-actin/CMV/*B*-globin intron hybrid promoter (LV-BDNF). The control virus expressed only the enhanced GFP tag under the same promoter (LV-GFP). The woodchuck hepatitis post-transcriptional response element was included in both.

Titers were determined by measurement of the p24 level in serial dilutions of the vector stock by an enzyme linked immunosorbent assay. Infectious units (IU) were determined by infection of 293 cells with serial dilutions of the vector stock and correlated with the p24 titers. Lentiviral titers were 0.5 to $1.0x10^9$ IU/mL (286 *u*g/mL p24) for the BDNF expressing virus and 0.5 to $1.0x10^9$ IU/mL for the GFP expressing virus (326 *u*g/mL p24).

Surgery: Complete transection

Rats were deeply anesthetized with a mixture (3 ml/kg) of ketamine (25 mg/ml), rompun (1.3 mg/ml), and acepromazine (0.25 mg/ml), then received complete T10 transection lesions (n = 6) (Blesch et al., 2003). Complete transections were used to detect descending axonal growth responses from all lesioned systems in the injured spinal cord that could transport BDA.

To create complete transections, the spinal cord was exposed at T10 and a gap of 2 mm in the spinal cord was created using fine scissors combined with aspiration with a fire-polished, fine-tipped suction pipette. Complete transection was visually verified using an operating microscope, with close inspection of all lateral and ventral components of the spinal canal to ensure that they were free of spared tissue. Eight subjects received scaffold implants the size of the complete transection (3x4x2mm ellipse). The scaffold channels were pre-filled with BDNF-secreting marrow stromal cells. Additionally, all animals received a BDNF lenti-virus injection 1.5mm caudal from the lesion. A quantity of 0.5*u*l was injected at two depths of 1 and 1.5mm. Animals with complete transections were sacrificed 4 month postlesion. For postoperative care, animals received 3 ml of Ringer's lactate subcutaneously twice daily for 3 days, manual bladder evacuation for 10 days, and 25 mg ampicillin twice daily for 2 weeks.

Histology

Animals received injections of the anterograde tracer biotinylated dextran amine (BDA) to trace the descending tracts (Weidner et al., 2001). One *u*l of

Biotinylated Dextran Amine (BDA, Mr 10,000, Molecular Probes), in a concentration of 100nl of 10%, was injected 2mm rostral to the complete transection, 1.5mm deep, in two sites using a PicoSpritzer II. Three days later, animals were transcardially perfused with a 4% solution of paraformaldehyde, postfixed overnight, and then transferred to a 30% sucrose solution for 3 days. Spinal cords were sectioned in the sagittal plane at 35-um intervals in blocks of 15 mm length at, and distal to, the T10 lesion site. To preserve the sensitive tissue architecture within the scaffolds, sections were mounted on slides. Immunocytochemical labeling for BDA was performed first with streptavidin–HRP-based light-level immunohistochemistry using direct mounted sections in the following protocol: (1) 1 h incubation with avidin-biotinylated peroxidase complex (1:150; Vector Elite kit; Vector Laboratories) at room temperature; and (2) treatment for 10 min with 0.05% solution of 3,3-diaminobenzidine, 0.01% H2O2, and 0.04% nickel chloride at room temperature. To examine lesion extent and graft survival, Nissl stains were performed in series of 1-in-7 sections in all animals. Lesions were considered adequate on Nissl stain if they completely transected the spinal cord.

Quantification of BDA labeled axons entering and exiting the scaffold

To determine the number of BDA-labeled axons that enter and exit the scaffold implanted into the complete transection of the spinal cord, two out of eight 35-um sagittal spinal cord BDA-labeled sections were examined under transmission light microscopy by an observer blinded to group identity. BDA-labeled axon profiles were

measured entering and exiting the scaffold in each channel. BDA labeled axon profiles were counted crossing a vertical line, sequentially placed every 200-microns to span a 2mm distance across the scaffold or the control lesion. A calibrated reticule eyepiece was used to delineate the vertical line and count crossing axon profiles at the scaffold edge of each channel at 20x and 40x magnification. Axon retention across the 2mm lesion were fit to a curve with an R-squared greater than 0.95. The average number of axons entering and exiting the scaffold were compared using ANOVA and a post hoc t-test.

Quantification of cellular orientation within the scaffold

To determine the cellular orientation over long distances (2mm), Nissl stained cells were examined with a 40x microscope over the entire length of six full treatment scaffold animals and six full treatment non-scaffold animals (wire knife, similar too Taylor et al., 2006). For each animal, six randomly sampled images within the lesion site were taken using PictureFrame and imported to ImageJ for angle measurements of the Nissl stained cellular architecture. Cells with an elliptical shape were examined, the orientation of the two ends of the ellipse were noted with reference to channel walls and dorsal lesion boundary for the full treatment scaffold and non-scaffold animals, respectively. Multiple sections were analyzed within the GFAP graft boundary for each animal. A 360 degree distribution of cellular orientation was calculated using the averages of each group. The distributions were compared using ANOVA and a post hoc t-test.

Quantification of BDA axon orientation with and without a scaffold

To determine the axon orientation over a 2mm distance, BDA stained cells were examined with a 40x microscope over the entire length of six full treatment scaffold animals and six full treatment non-scaffold animals (wire knife, similar too Taylor, 2006). For each animal, six randomly sampled images within the lesion site were taken using PictureFrame and imported to ImageJ for angle measurements of the BDA stained axon architecture. Axon profiles were approximated with a series of 100um lines, the orientation of the two ends of the lines were noted with reference channel walls and dorsal lesion boundary for the full treatment scaffold and non-scaffold animals, respectively. Multiple sections were analyzed within the GFAP graft boundary for each animal. A 360 degree distribution of axon orientation was calculated using the averages of each group. The distributions were compared using Anova and a post hoc t-test.

Results

A linearized agarose scaffold enhances the number of descending axons reaching the distal end of a 2mm lesion

A combinatorial treatment of BMSC-BDNF and Lenti-BDNF, supported axon growth into and beyond a highly linearized agarose scaffold in a complete spinal cord transection site (Fig. 3.1, 3.2). Injected rostral to the lesion, biotinylated dextran

amine (BDA) showed axons crossing into the rostral side and exiting the caudal end of the scaffold. The combinatorial BDNF treatment with the complete transection scaffold had 78 $\pm$ 7 BDA labeled axons entering and 66 $\pm$ 7 BDA labeled axons exiting each channel. However, the cell suspension combinatorial treatment had an average of 63 $\pm$ 14 axons entering the lesion site with 0 $\pm$ 0 BDA axons exiting (Fig. 3.3, 3.4). Furthermore, in the lesion only control, there were no axons entering or exiting the lesion. While crossing a 2 mm lesion, the combinatorial BDNF treatment with a scaffold exhibited 85 $\pm$14% BDA axon retention across the full length of the lesion, while the cell suspension control had a 0% retention. Therefore, the scaffold significantly and substantially increased the number of descending axons reaching the distal aspect of a complete transection.

The reduction in mechanical tortuousity could potentially increase the number of axons crossing the entire length of the lesion site by constraining the regenerating axons to linear arrays within the lesion. To determine the number of axons retained every 200*u*m, BDA labeled axon profiles were counted crossing a vertical line, sequentially placed every 200-microns across the 2mm distance of the scaffold or the control lesions (Fig. 3.5). The overall number of descending axons retained over the distance of the lesion was maintained in a linear manner in the full treatment scaffold, while it sharply decayed in non-scaffold lesions of $y = 0.06x^2 - 0.19x + 1.00$ ($R^2 = 0.96$) and $y = -0.60x^2 - 0.32x + 1.00$ ($R^2 = 0.99$), respectively. Furthermore, there were no axons crossing the cystic cavity within the lesion only control.

Linearized channels enhance cellular orientation within a 2mm long complete transection

We measured cellular orientation within lesion sites using a Nissl stain for cell bodies in six full treatment animals with and without scaffolds. All cells were approximated as ellipses, where the two longest ends were considered the end points for angle measurement of cellular orientation. Linear was defined as 90 $\pm$ 5 degrees parallel to the rostral-caudal axis of the spinal cord (Fig. 3.6). Within the scaffold, approximately 80 $\pm$ 2% of the cells were oriented in a linear manner, while without the scaffold only 6 $\pm$ 2% were oriented in a linear manner. The cellular linearity within the scaffold treatment group was greater in than in the non-scaffold treatment group (t-test, $p < 0.05$). Therefore, the scaffold channels promoted a highly linear cellular environment within the lesion site, which potentially enhanced axonal guidance by reducing mechanical tortuousity across the large lesion site.

The combinatorial treatment of growth factor delivery and linearized channels enhanced axon orientation within a 2mm long complete transection

Descending axon orientation was examined by injection of Biotinylated Dextran Amine (BDA) rostral to the spinal cord transection site. Axons were approximated as 100*u*m lines, and the angle of orientation of the axon segment were measured with reference to the angle of channel walls, or, in cell suspension graft animals, to the longitudinal axis of the spinal cord. Linearity was defined as 90 $\pm$ 15 degrees parallel to the spinal column (Fig. 3.6). Within the scaffold, approximately 85 $\pm$ 4% of the

axons were oriented in a linear manner, where only 16.5 + 4% of axons were linearly oriented in cell suspension grafts (t-test, $p < 0.05$). Therefore, the combinatorial treatment of the growth factor gradient and the agarose scaffold channels enhanced the descending axon orientation within a complete transection.

In addition, the scaffold elicited a minimal inflammatory response. As shown by neurofilament immunolabeling (NF), short-tract axons entered each end of the scaffold, indicating that both ends of the lesion boundary permitted axon growth into the lesion. Additionally, the scaffold supported Schwann cell (S100) migration into the lesion, thus potentially providing a possible source for axon myelination (Fig. 3.7). As previously reported (Stokols et al., 2006), only rare inflammatory cells were present within scaffolds, reflected by astrocyte (GFAP) and microglial (IBA) immunolabeling (Fig. 3.7), illustrating the biocompatibility of the scaffold.

Regenerating descending axons do not re-penetrate host spinal cord beyond lesion

Although descending axons guided by the linearized scaffold reached the distal end of the complete transection, they did not penetrate or regenerate into the host parenchyma beyond the lesion. Similarly to our previous study in the ascending system, axons filled the cell layer that was interposed between the distal aspect of the scaffold implant and the distal spinal cord. The reactive cell layer had a mean width of 300$\pm$200*u*m from the end of the scaffold host spinal cord. The Nissl stain revealed a cell layer consisting of cells with ultrastructural characteristics of leptomeningeal fibroblasts (Fig. 3.8). Additionally, GFAP immunoreactivity indicated the border

between the reactive cell layer and host parenchyma was demarcated by the immunoreactive astrocytic processes (Fig. 3.8). Furthermore, there was a distinct border along the reactive cell layer/host parenchymal interface composed of the inhibitory extracellular matrix marker NG2 (Fig.3.8).

Discussion

Findings of the present study indicate that a linearized scaffold and a combinatorial BDNF treatment enhanced the number of descending axons reaching the distal end of a complete transection lesion when compared to a BDNF combinatorial treatment with a disorganized cell suspension. Furthermore, both groups with the BDNF combinatorial group supported axon penetration into the graft, which was not supported in the cystic lesion cavities of the lesion-only controls. Similar to previous findings in the ascending sensory system, we found that the cellular and axonal orientation within the spinal cord was highly linear when compared to the disorganized cellular suspension graft. However, descending axons reaching the distal aspect of the scaffold did not repenetrate the host parenchyma. The scaffold/host interface along the complete transection consisted of a reactive cell layer which prevented axons from regenerating into the host parenchyma.

Combinatorial treatments enhanced axon growth into the complete transection when compared to the lesion only-control, which formed a cystic cavity. Methods to

fill lesion cavities often include cellular grafts (Ronsyn et al., 2008). Thus. it is likely that the cellular graft component of the BDNF combinatorial approach reduced the formation of the cystic lesion. Furthermore, we showed that the addition of a cell seeded linearized scaffold to the complete transection enhanced the cellular microarchitecture within the complete transection to more closely mimic the innate linearity of the intact spinal cord. Consequently, using methods to mimic the innate linearity of the intact spinal cord, while reducing cystic formation, we may further enhance guidance of axonal regeneration across a large lesion site.

Although, the BDNF combinatorial treatment with a disorganized cell suspension promoted descending axon growth into the lesion, the number of axons reaching the distal aspect of the lesion was significantly enhanced when adding the linearized scaffold to the treatment. Thus, one might ask: by what mechanism does the linearized scaffold enhance the number of regenerating axons reaching the distal aspect of the lesion? Mechanistically, the axonal guidance may be enhanced through mechanical guidance, by cellular orientation or physical constraints of the scaffold walls, through the lesion cavity. Specifically, the scaffold channels may be constraining axons into linear arrays, therefore; 1) decreasing the physical length axons travel to cross the lesion 2) therefore increasing the rate they reach the distal end 3) while consequently reducing the overall energy used in the axonal extension mechanisms. Therefore, due a more linear path and axonal orientation (receptors alignment may more readily detect a gradient), altered transient growth state (inhibitory aspects of environment may be less prominent early on), and a larger

source of potential energy (neurons used less energy on cytoskeletal extension), the regenerating axon may respond more readily to a growth factor gradient. Additionally, or alternatively, the growth factor gradient may diffuse more uniformly through a series of linearly arranged spheres (cells) than in a disorganized graft, thus eliciting an enhanced axonal response via a more easily detectable growth factor gradient. After comparing the test groups, it is a possibility that a BDNF combinatorial treatment and a linearized agarose scaffold mechanistically enhance axon growth in a synergistic manner.

Although the reactive cell layer was present at both ends of the scaffold implant, BDA and neurofilament axons were not prevented from axon penetration into the scaffold. However, as mentioned above, the BDA axons did not regenerate beyond the reactive cell layer and into the caudal host tissue. One possible explanation is that the cell layer was formed after axons regenerated into the lesion site. Another potential explanation for this may be that the reactive cell layer increased the inhibitory properties on a time scale that only affected the axons reaching the distal aspect of the lesion. A third, biomechanical view, is that due to cell deposition and the swelling along the lesion boundary, axon navigation into the lesion site may encounter a cellular architecture that represents a mechanical “funnel”, while axons attempting to re-enter the host parenchyma may encounter a swollen, flat surface with only few entry points. Although, the axons did not reach the distal aspect of the lesion in the cell suspension graft, a reactive cell layer did not appear to be present. Therefore, the formation of the reactive cell layer between the host spinal cord parenchyma and the

agarose scaffold supports axonal emergence from scaffolds; however, it does not facilitate axon regeneration beyond the lesion.

Future studies will determine whether the scaffold, when combined with a method to permit axon growth beyond the inhibitory environment of the lesion/host interface, may enhance the potential for descending axon reinnveration across large lesions. Future combinatorial treatments to enhance axon growth beyond the inhibitory environment may include the unidirectional release of growth factors sealed within the agarose scaffold with a PLGA coating (such as PLGA, Vozzi et al. 2003; Holy et al., 2000; Karp et al., 2003). Potential combinatorial treatments to minimize the formation of the reactive cell layer which may include the use of a scaffold with an elasticity modulus more closely mimicking the spinal cord, such as the 0.6% agarose concentrations (Discussed in next chapter). To reduce cellular migration between the host wall and the scaffold interface, the scaffold tips may be coated with poly(ethylene glycol) (PEG) to promote cellular solubility along the host interface (Shi et al., 1999; Norwood et al., 1976; Lentz, 1994). Furthermore, to prevent fibroblast growth, an antimitotic may be delivered through the scaffold or released via an Alzet pump (Khaw et al., 1994; Bach, 1953; Schiff and Horwitz, 1980). Thus, it is essential that further development of the scaffold technology focus on limiting the reactive cell matrix that forms between the scaffold and host spinal cord, to determine whether the greater pool of axons presented to the distal aspect of the lesion will result in a greater number of axons regenerating into the host spinal cord *beyond* the lesion site.

Acknowledgement

Chapter 3 is original work in preparation as Gros TR, Sakamoto J, Blesch A, Tuszynksi MH. A physical and chemical combinatorial therapy enhances descending axon guidance across a complete spinal cord transection and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

References

Azizi, S, D Stokes, B Augelli, C Digirolamo and D Prockop (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats-similarities to astrocyte grafts. Proceedings of the National Academy of Sciences 95(7): 3908-13.

Bach, S and I Simon-Reuss (1953) Arginase, an antimitotic agent in tissue culture. Biochim Biophys Acta 11: 396-402.

Blesch, A and M Tuszynski (2003) Cellular gdnf delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination. The Journal of Comparative Neurology 467(3): 403-17.

Blesch, A (2004) Lentiviral and mlv based retroviral vectors for ex vivo and in vivo gene transfer. Methods 33(2): 164-72.

Bradbury, E, S Khemani, R Von, J Priestley and S Mcmahon (1999) Nt-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. European Journal of Neuroscience 11(11): 3873-83.

Bunge, M (1994) Transplantation of purified populations of schwann cells into lesioned adult rat spinal cord. Journal of Neurology 242: 36-39.

Bunge, M and N Kleitman (1997). Schwann cells as facilitators of axonal regeneration in cns fiber tracts. Altschul Symposium Proceedings.

Cheng, H, Y Cao and L Olson (1996) Spinal cord repair in adult paraplegic rats: Partial restoration of hind limb function. Science 273(5274): 510.

David, S and A Aguayo (1981) Axonal elongation into peripheral nervous system" bridges" after central nervous system injury in adult rats. Science 214(4523): 931.

Dontchev, V and P Letourneau (2002) Nerve growth factor and semaphorin 3a signaling pathways interact in regulating sensory neuronal growth cone motility. Journal of Neuroscience 22(15): 6659.

Fields, R, J Le Beau, F Longo and M Ellisman (1989) Nerve regeneration through artificial tubular implants. Prog Neurobiol 33(2): 87-134.

Fung, Y (1993). Biomechanics: Mechanical properties of living tissues, Springer.

Gallo, G, F Lefcort and P Letourneau (1997) The trka receptor mediates growth cone turning toward a localized source of nerve growth factor. Journal of Neuroscience 17(14): 5445-54.

Genç, B, P Özdinler, A Mendoza and R Erzurumlu (2004) A chemoattractant role for nt-3 in proprioceptive axon guidance. PLoS Biol 2(12): e403.

Gundersen, R and J Barrett (1979) Neuronal chemotaxis: Chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206(4422): 1079-80.

Holy, C, C Cheng, J Davies and M Shoichet (2000) Optimizing the sterilization of plga scaffolds for use in tissue engineering. Biomaterials 22(1): 25-31.

Houweling, D, A Lankhorst, W Gispen, P Bär and E Joosten (1998) Collagen containing neurotrophin-3 (nt-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. Experimental Neurology 153(1): 49-59.

Karp, J, M Shoichet and J Davies (2003) Bone formation on two-dimensional poly (dl-lactide-co-glycolide)(plga) films and three-dimensional plga tissue engineering scaffolds in vitro. Journal of Biomedical Materials Research Part A 64(2): 388-96.

Khaw, P, N Occleston, G Schultz, I Grierson, M Sherwood and G Larkin (1994) Activation and suppression of fibroblast function. Eye(London. 1987) 8: 188-95.

Kirik, D, C Rosenblad and A Bjorklund (2000) Preservation of a functional nigrostriatal dopamine pathway by gdnf in the intrastriatal 6-ohda lesion model depends on the site of administration of the trophic factor. European Journal of Neuroscience 12(11): 3871-82.

Kwon, B, J Liu, C Lam, W Plunet, L Oschipok, W Hauswirth, A Di Polo, A Blesch and W Tetzlaff (2007) Brain-derived neurotrophic factor gene transfer with adeno-associated viral and lentiviral vectors prevents rubrospinal neuronal atrophy and stimulates regeneration-associated gene expression after acute cervical spinal cord injury. Spine 32(11): 1164.

Langer, R and J Vacanti (1993) Tissue engineering. Science 260(5110): 920-26.

Lentz, B (1994) Polymer-induced membrane fusion: Potential mechanism and relation to cell fusion events. Chem Phys Lipids 73(1-2): 91-106.

Letourneau, P (1978) Chemotactic response of nerve fiber elongation to nerve growth factor. Dev Biol 66(1): 183-96.

Liu, Y, B Himes, J Solowska, J Moul, S Chow, K Park, A Tessler, M Murray, E Snyder and I Fischer (1999) Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. Experimental Neurology 158(1): 9-26.

Liu, Y, D Kim, B Himes, S Chow, T Schallert, M Murray, A Tessler and I Fischer (1999) Transplants of fibroblasts genetically modified to express bdnf promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. Journal of Neuroscience 19(11): 4370-87.

Lu, P, L Jones, E Snyder and M Tuszynski (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Experimental Neurology 181(2): 115-29.

Lu, P, H Yang, L Jones, M Filbin and M Tuszynski (2004) Combinatorial therapy with neurotrophins and camp promotes axonal regeneration beyond sites of spinal cord injury. Journal of Neuroscience 24(28): 6402-09.

Lu, P, L Jones and M Tuszynski (2005) Bdnf-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Experimental Neurology 191(2): 344-60.

Marchand, R, S Woerly, L Bertrand and N Valdes (1993) Evaluation of two cross-linked collagen gels implanted in the transected spinal cord. BRAIN RESEARCH BULLETIN 30: 415-15.

Naldini, L, U Blomer, F Gage, D Trono and I Verma (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proceedings of the National Academy of Sciences 93(21): 11382-88.

Nikulina, E, J Tidwell, H Dai, B Bregman and M Filbin (2004) The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proceedings of the National Academy of Sciences 101(23): 8786-90.

Norwood, T, C Zeigler and G Martin (1976) Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. Somatic Cell and Molecular Genetics 2(3): 263-70.

Oudega, M, S Varon and T Hagg (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: Promoting effects of conditioning lesion and graft predegeneration. Exp Neurol 129(2): 194-206.

Oudega, M and T Hagg (1999) Neurotrophins promote regeneration of sensory axons in the adult rat spinal cord. Brain Research 818(2): 431-38.

Palsson, B (2003). Tissue engineering, CRC Press.

Paves, H and M Saarma (1997) Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. Cell and Tissue Research 290(2): 285-97.

Pearse, D, F Pereira, A Marcillo, M Bates, Y Berrocal, M Filbin and M Bunge (2004) Camp and schwann cells promote axonal growth and functional recovery after spinal cord injury. Nature Medicine 10: 610-16.

Pfeifer, A, M Ikawa, Y Dayn and I Verma (2002) Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proceedings of the National Academy of Sciences 99(4): 2140.

Quencer, R and R Bunge (1996) The injured spinal cord: Imaging, histopathologic, clinical correlates, and basic science approaches to enhancing neural function after spinal cord injury. Spine 21(18): 2064.

Ratner, B (1997). Biomaterials science: An introduction to materials in medicine, Academic Press.

Ringstedt, T (1997) Limb proprioceptive deficits without neuronal loss in transgenic mice overexpressing neurotrophin-3 in the developing nervous system. Development 124(13): 2603-13.

Romero, M, N Rangappa, M Garry and G Smith (2001) Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. Journal of Neuroscience 21(21): 8408.

Ronsyn, M, Z Berneman, V Van Tendeloo, P Jorens and P Ponsaerts (2008) Can cell therapy heal a spinal cord injury? Spinal Cord.

Schiff, P and S Horwitz (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proceedings of the National Academy of Sciences 77(3): 1561-65.

Shi, R, R Borgens and A Blight (1999) Functional reconnection of severed mammalian spinal cord axons with polyethylene glycol. JOURNAL OF NEUROTRAUMA 16(8).

Stokols, S, J Sakamoto, C Breckon, T Holt, J Weiss and M Tuszynski (2006) Templated agarose scaffolds support linear axonal regeneration. Tissue Engineering 12(10): 2777-87.

Tator, C and M Fehlings (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75(1): 15-26.

Taylor, L, M Tuszynski and A Blesch (2004). Nt-3 gradients generated by lentiviral gene delivery promote axonal bridging into and beyond sites of spinal cord injury. Soc. Neurosci. Abstr.

Tessarollo, L, V Coppola and B Fritzsch (2004) Nt-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea. Journal of Neuroscience 24(10): 2575-84.

Tucker, K, M Meyer and Y Barde (2001) Neurotrophins are required for nerve growth during development. Nature Neuroscience 4: 29-37.

Tuszynski, M, K Gabriel, F Gage, S Suhr, S Meyer and A Rosetti (1996) Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. Experimental Neurology 137(1): 157-73.

Vozzi, G, C Flaim, A Ahluwalia and S Bhatia (2003) Fabrication of plga scaffolds using soft lithography and microsyringe deposition. Biomaterials 24(14): 2533-40.

Weidner, N, A Ner, N Salimi and M Tuszynski (2001) Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences 98(6): 3513.

Woerly, S, P Petrov, E Sykova, T Roitbak, Z Simonova and A Harvey (1999) Neural tissue formation within porous hydrogels implanted in brain and spinal cord lesions: Ultrastructural, immunohistochemical, and diffusion studies. Tissue Eng 5(5): 467-88.

Woerly, S (2000) Restorative surgery of the central nervous system by means of tissue engineering using neurogel implants. Neurosurgical Review 23(2): 59-77.

Xu, X, V Guénard, N Kleitman, P Aebischer and M Bunge (1995) A combination of bdnf and nt-3 promotes supraspinal axonal regeneration into schwann cell grafts in adult rat thoracic spinal cord. Experimental Neurology 134(2): 261-72.

Zhang, Y, P Dijkhuizen, P Anderson, A Lieberman and J Verhaagen (1998) Nt-3 delivered by an adenoviral vector induces injured dorsal root axons to regenerate into the spinal cord of adult rats. J Neurosci Res 54(4): 554-62.

Zhou, L, B Baumgartner, S Hill-Felberg, L Mcgowen and H Shine (2003) Neurotrophin-3 expressed in situ induces axonal plasticity in the adult injured spinal cord. Journal of Neuroscience 23(4): 1424.

Zweifel, L, R Kuruvilla and D Ginty (2005) Functions and mechanisms of retrograde neurotrophin signalling. Nat Rev Neurosci 6(8): 615-25.

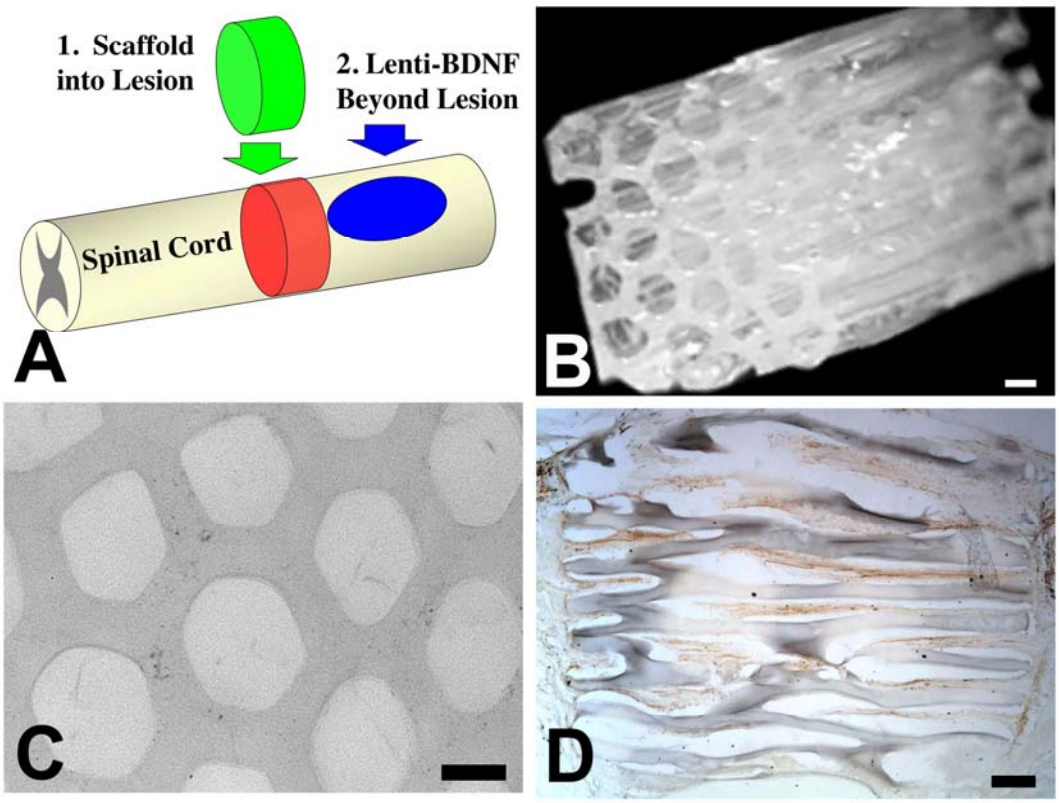

Fig. 3.1 The linearized scaffold is implanted into a complete transection site to guide axons across a 2mm lesion distance. A) The lesion paradigm consists of a 2mm scaffold implanted into the complete T10 lesion, bone marrow stromal cells genetically modified to secrete BDNF are filled into the scaffold channels to attract descending axons into the lesion, and a growth factor (BDNF) is expressed using lentiviral gene delivery beyond the lesion site, to stimulate growth of descending axons beyond the lesion. B) A phase contrast image shows the longitudinal orientation of the linearized channels in a scaffold. C) A nissl stain of scaffold shows consistent scaffold architecture with 100um wall thickness and 200um channels. D) The scaffold is shown in the complete transection site, and is integrated anatomically. Scale Bars B) 200um, C) 100um, D) 250um.

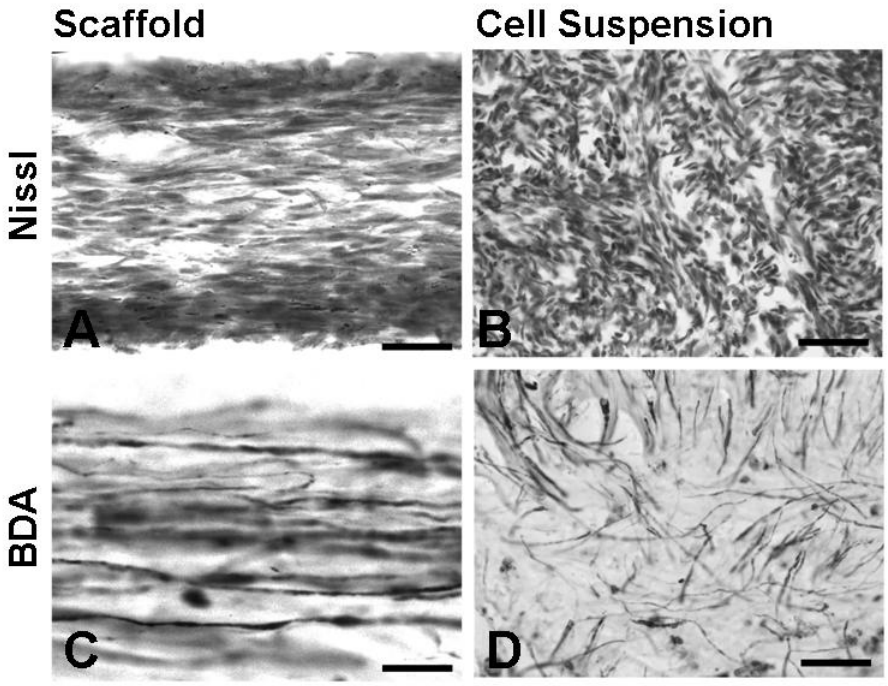

Fig. 3.2 The scaffold promotes cellular and descending axonal linearity within the complete transection lesion site. A) A nissl stain in a complete T10 transection shows cellular linearity within a scaffold channel and B) lack of cellular linearity in a cell graft treatment. C) A BDA stain in a complete T10 transection shows axon linearity within a scaffold channel and D) lack of axon linearity in a cell graft treatment. Scale bars A-D) 200um.

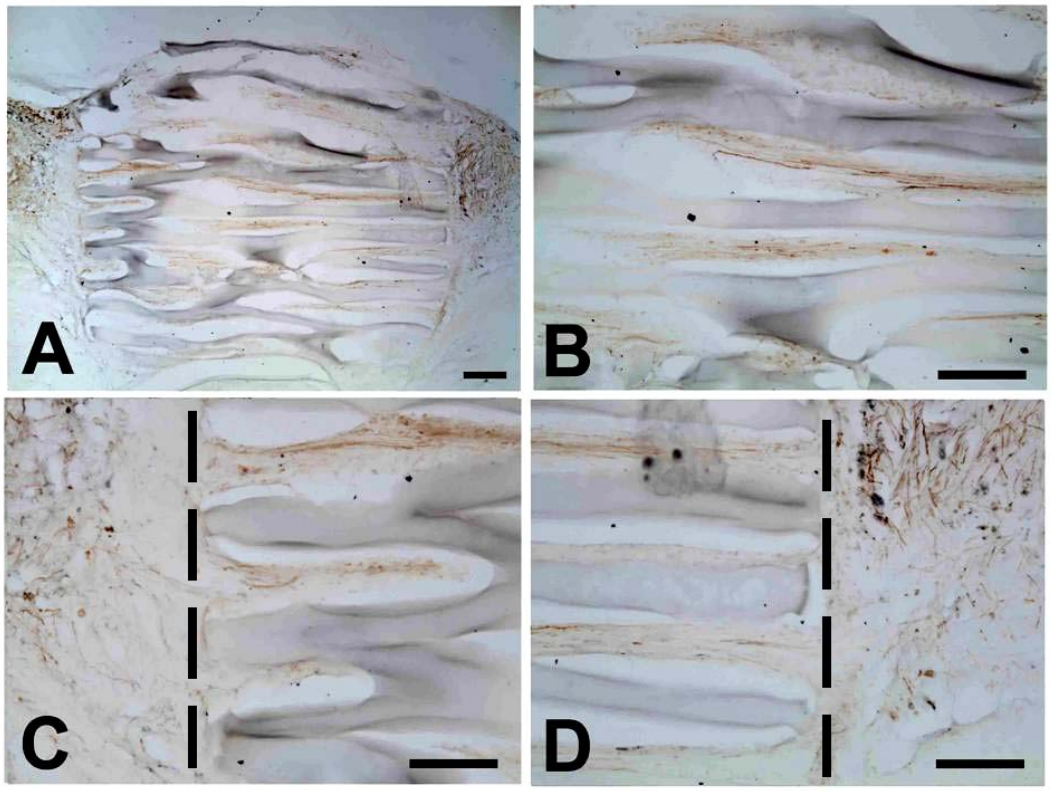

Fig. 3.3 Scaffold implant into complete transection guides BDA labeled descending axons across a 2mm distance of the lesion. A) BDA labeled descending axons cross the entire scaffold length of the complete transection. BDA labeled descending axons are found within the B) center, C) entering, and D) exiting the scaffold. The black vertical lines demarcated where descending BDA labeled axons were quantified. C) entering and D) exiting the scaffold. Scale bars A,C,D) 250*u*m B) 100.

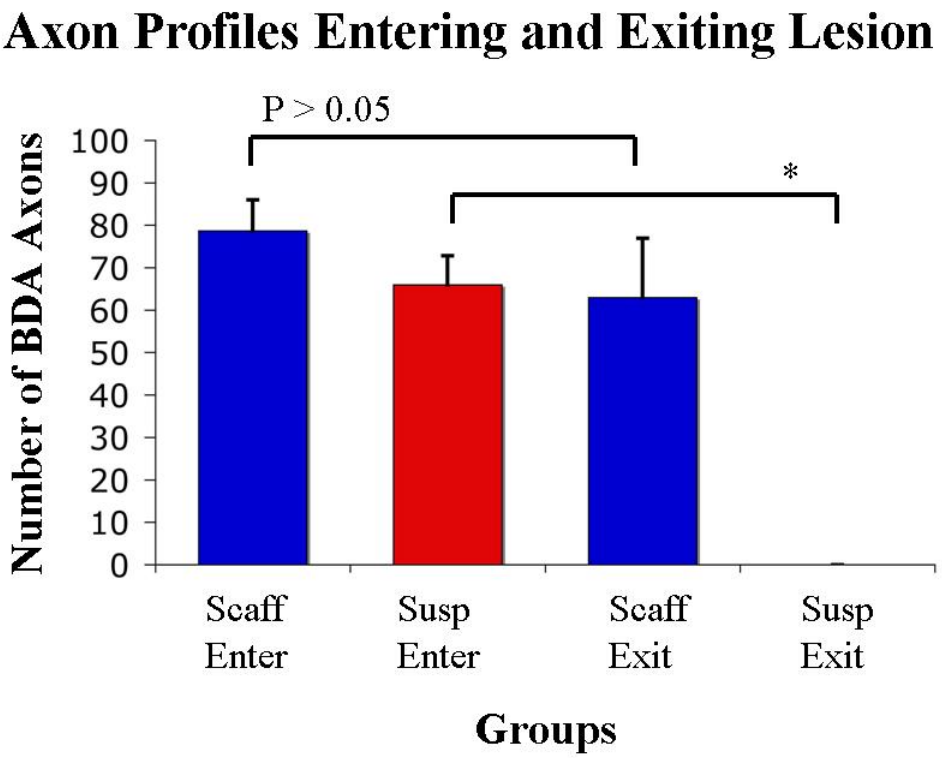

Fig. 3.4 BDA labeled axons enter and exit the scaffold with a statistically similar retention compared to the cell suspension. The number of axons exiting the scaffold is in the scaffold is statistically similar (t-test $p>0.05$), while the number of axons exiting the cell suspension graph is statistically different than those that entered (*, discretely different).

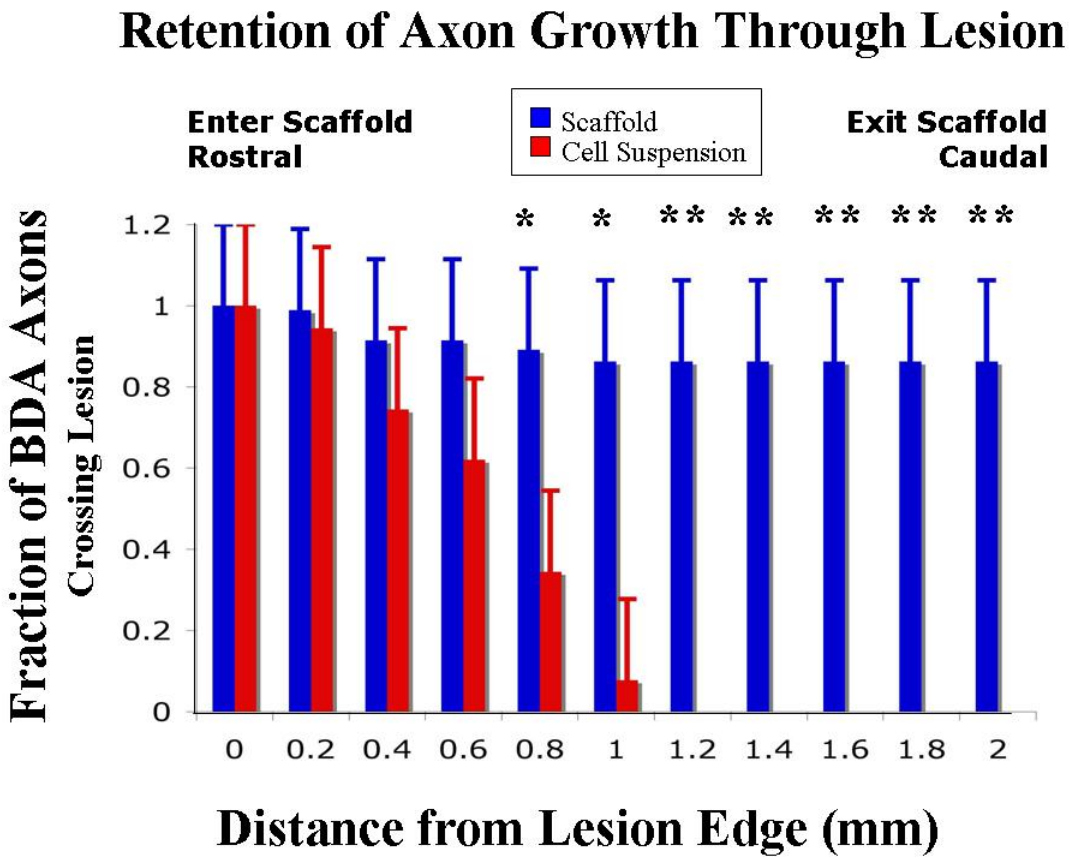

Fig. 3.5 Descending axon retention in a complete transection with and without a scaffold. The BDA labeled axons cross the entire scaffold distance when guided within a linear scaffold, while axons within the cell graft lesion diverge along a tortuous pathway and do not extend across the 2mm lesion distance. T-tests were performed (*, $p < 0.05$). (**, indicated groups that were substantially different due to a zero comparison).

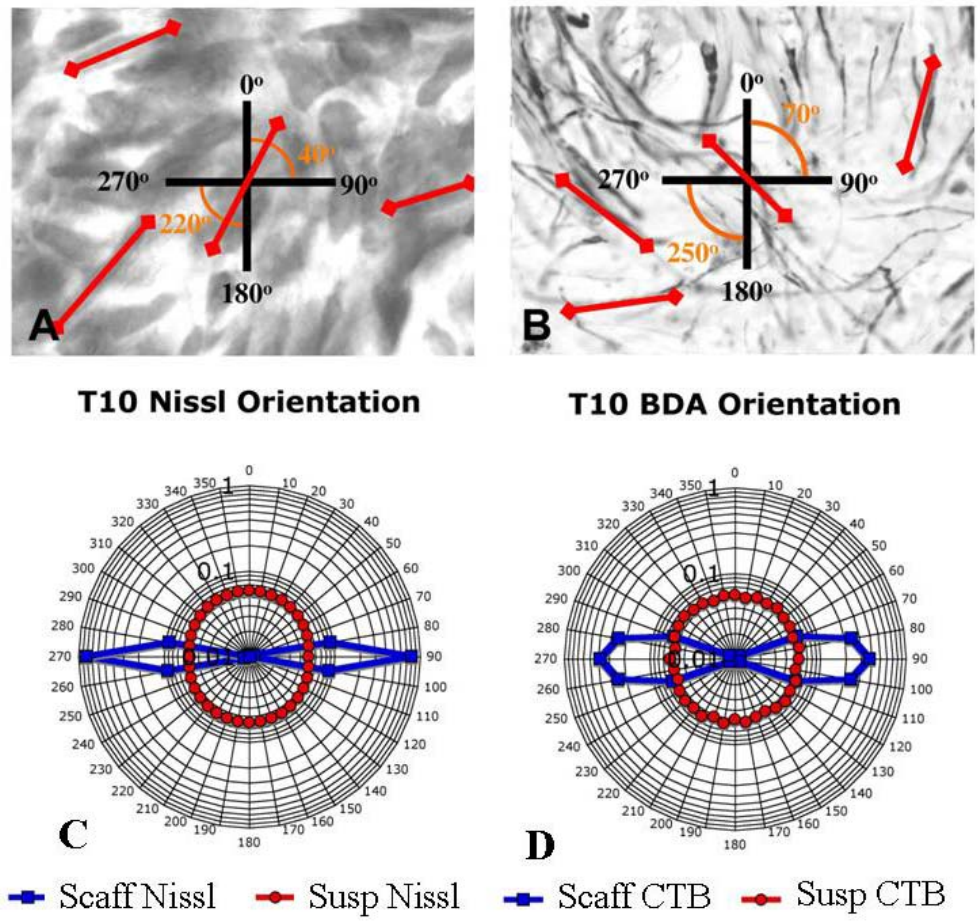

Fig. 3.6 Methods and qauntification of nissl and descending axon linearity with and without a scaffold in a complete transection. A) Nissl cells were approximated as ellipses and the two ends were measured with respect to the dorsal aspect. B) BDA labeled axons were averaged into 100um segments and the angles were measured with respect to the dorsal aspect. C) Nissl Orientation within the complete transection was highly linear within the scaffold compared to the disorganized cells within the non-scaffold lesion. D) BDA labeled axons were in a highly linear manner within the scaffold, compared to the highly disorganized BDA axons within a non-scaffold lesion.

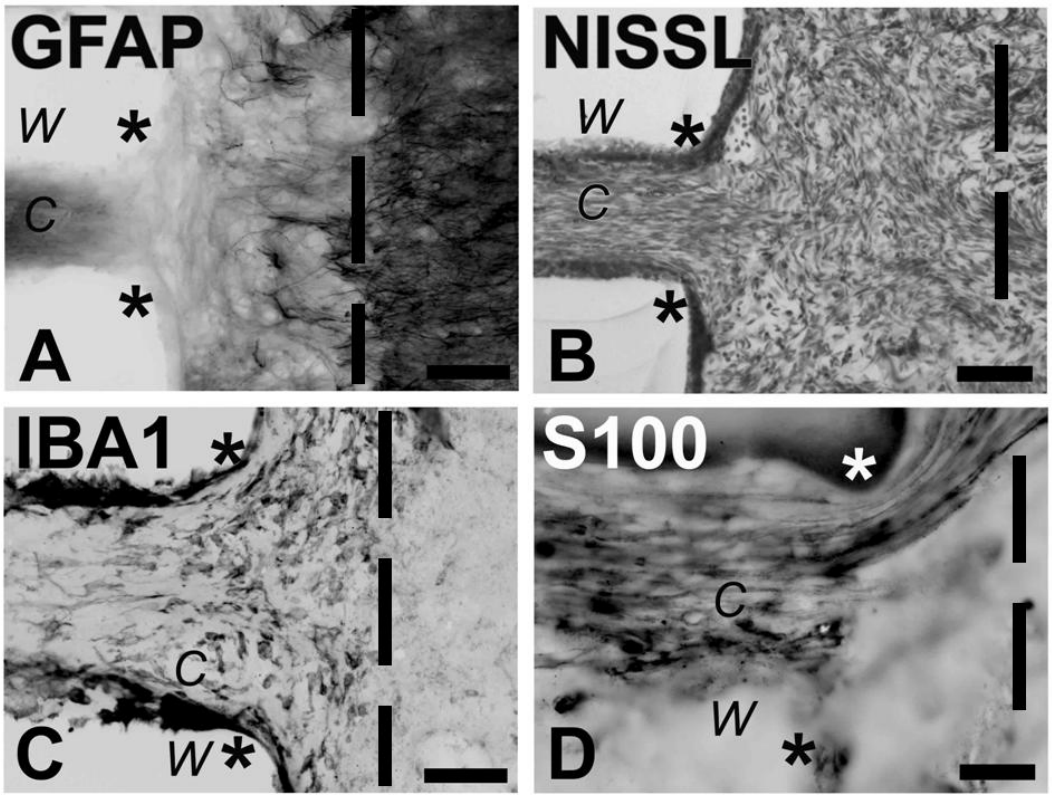

Fig. 3.7 The scaffold host interface supports axon permissive infrastructure. A) GFAP indicates no astrocytes within the scaffold channels. B) Granularized tissue was minimal, as indicated from the Nissl stain. C) IBA1 labeled a minimal amount of migroglial cells within the scaffold. D) Schwann cells migrated into the scaffold, providing a potential means for mylinating axons. Channel walls indicated by *W* and cell fill by *C*. The scaffold edge is delineated by an asterisk(*****). The vertical black dashed lines represent the interface of the reactive cell layer with the host spinal cord. Scale bars A,B)100um C,D) 50um.

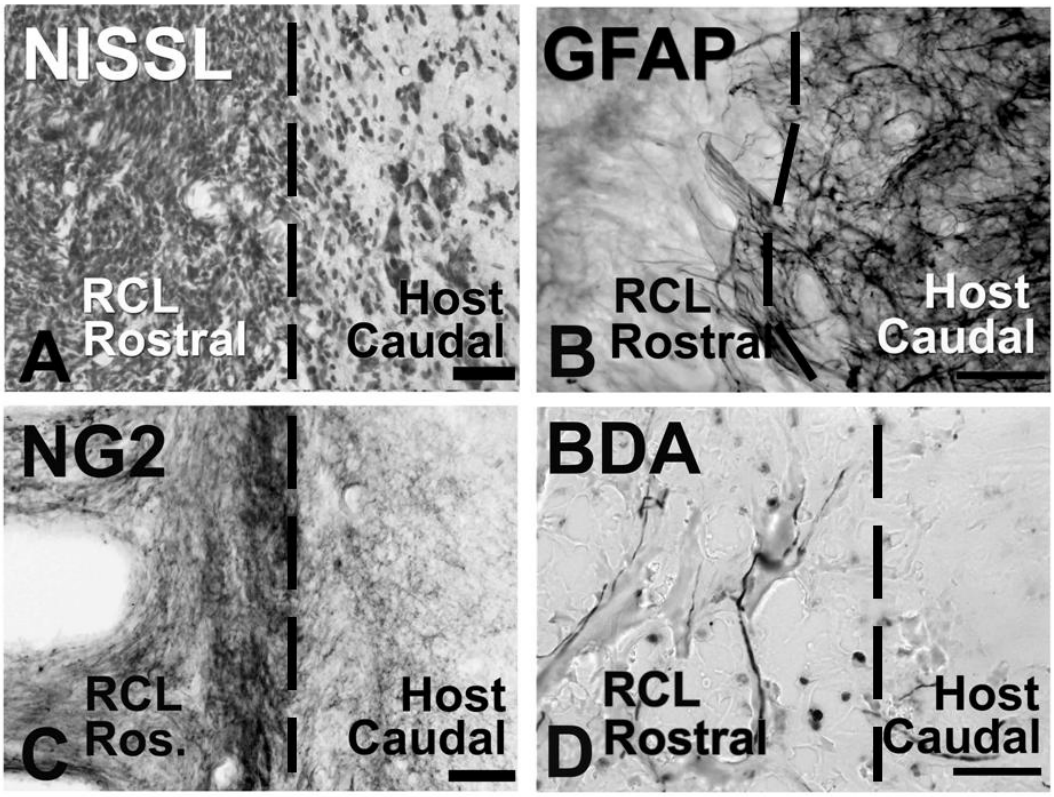

Fig. 3.8 The reactive cell layer adjacent to the scaffold implant. Sections are cut in a sagittal plane; rostral is to left, caudal to right. Caudal host tissue is not permeated by descending axons due to the reactive cell layer. A) Nissl stained cells are spaced evenly and with lower density in the host tissue, while the nissl stained cells are in a high density along the reactive cell layer. B) Host astrocytic processes (GFAP label) are evident in host spinal cord but exhibit limited extension into reactive cell layer. C) Immunolabeling for the chondroitin sulfate proteoglycan molecule NG2 is increased in the region of the reactive cell layer and host parenchyma adjoining the lesion cavity forms along the reactive cell layer. D) BDA axons turn dorsally within the reactive cell layer and do not re-penetrate host tissue. The black dashed line delineates the separation of reactive cell layer (right) from the rostral host tissue (left). Scale bars A-C) and D) 50 um.

CHAPTER 4

A highly linearized and mechanically scalable nerve conduit for peripheral nerve regeneration

Abstract

Axonal regeneration naturally occurs with relative efficiency in the peripheral nervous system (PNS) across nerve gaps under 4mm, and fails entirely over injury distances exceeding 1 cm. Thus, there exists a need to identify methods for enhancing peripheral nerve regeneration. Conduits are clinically available for lengths greater than 4-mm (Neurogen), but have not been capable of functionally replacing the "gold standard" of peripheral nerve injury, the autografting of sural nerve into the injury gap. To mimic aspects of a nerve autograft we designed a conduit resembling the guiding microarchitecture of the endoneurial tubes found within a nerve autograft. We now show that a linearized agarose scaffold is biocompatible for 1 year. Additionally, we show that our scaffolds supported axonal growth to the distal aspect of the conduits, beyond the clinically relevant length of 4-mm. Furthermore, using methods to reduce surface tension and increase agarose diffusion, we scaled the scaffold to lengths beyond 1 cm, the recovery threshold of a nerve autograft. To reduce the time for a physical substrate, such as fibrin, to fill the empty lumen, we scaled the lumen area from 74% to 45% by altering the template structure. The scaffold strength was scaled using a novel superheating method to change agarose concentrations from 0.2% up to

14%, allowing for the potential to more closely mimic the mechanical properties of the nerve or increase of lumen strength to prevent collapse Furthermore, measurements of release kinetics indicated that proteins will rapidly diffuse through agarose, providing the basis for future designs of growth factor delivery throughout the conduit . Therefore, our current conduit design mimics several key characteristics of a nerve autograft.

Introduction

There are nearly 20,000 peripheral nerve procedures performed a year in the United States (Kelsey et al., 1997). Peripheral nerve injury (PNI) has a societal an economical impact as it results in a large number of nonfunctional or disability work days. Furthermore, 2.8% of peripheral injuries result in life-long disability (Noble et al., 1998).

After peripheral nerve injury, nerve ends are either sutured together, for lesions smaller than 4mm, or a nerve autograft is used to bridge longer distances (Yu, 2003; Lundborg, 1988; Mackinnon and Dellon, 1988). A bridge is needed for distances beyond 4mm to reduce tension that might inhibit nerve regeneration (Millesi, 1967). There are a few FDA approved nerve conduits which are used to bridge short distances such as “Nerve Guide” and “SaluBridge Nerve Cuff” (Archiblad et al., 1995; Lundborg et al., 1997). Although these conduits are normally constructed to replace a nerve autograft, there is a need to improve functional recovery if the defect extends

beyond1cm (Evans, 2001). Currently, bioengineering strategies for peripheral nerve repair focus on developing conduits for long (>1-cm) defects while attempting to accelerate axonal growth rates and functional recovery.

Hydrogel based nerve guidance channels have been shown to offer a biocompatible water-based interface, while providing microstructure guidance for cellular migration and regenerating axons. The current fabrication technology for hydrogels being used in tissue engineering applications is based on solvent-casting/particulate-leaching techniques, injection molding, fiber templating and freeze-drying (Hollander et al., 2004; Norman and Dsai, 2006). Nerve guidance channels have been predominantly fabricated as conduit shells, without internal structures, thus lacking guidance to cells and axons across a site of injury. More recently, scaffolds with internal structures to guide linear axonal growth either by fibers or open pores have been investigated using collagen or other biodegradable materials (Archbald et al., 1991; Sinis et al., 2007; Hadlock, 2000; Evans et al., 1999). However, many approaches result in poor fiber alignment and non-linear axonal growth.

In the present study, we aimed to design conduits mimicking the regeneration promoting features of a nerve autograft. We developed a method to produce a scalable hydrogel-based scaffold with highly linearized channels. The conduit contained channel sizes of 200μm and exhibited biocompatibility, vascularization, and permissiveness for axon growth beyond the clinically relevant length of 4mm. Channel length, lumen cross sectional area, and scaffold strength are readily modifiable for future testing to improve scaffold performance in the injury site. We

examined protein release from agarose into water and found that there was a rapid release, illustrating that the protein release was not limited from the internal diffusion of agarose (within a time scale of 1 hr). Therefore, upon implantation into a tissue the protein would readily diffuse through agarose while only being limited by the (external) diffusion of the host interface. Therefore, our current conduit design mimics several key characteristics of a nerve autograft.

Materials and methods

Scaffold core and sleeve manufacturing

The fabrication of the peripheral nerve conduit was based on a modification of our previously published methods to develop the linearized agarose scaffold (Stokols et al., 2006). Specifically, scaffolds were made using Multi-component fiber bundles (MCFBs) with 100um walls and 200 micron channels with a lumen cross sectional area of 45% (Paradigm Optics, Vancouver, WA) (Fig. 4.1). Additionally, a 46um wall and 278um channel scaffold was manufactured, generating an open luminal cross sectional area of 74% relative to wall thickness (Fig. 4.1). Fibers were arranged in a hexagonal close-packed array separated by a continuous matrix of poly(methyl methacrylate) (PMMA). MCFBs were simultaneously extruded and fused such that the polystyrene fibers were oriented parallel to the longitudinal axis of the bundles. Using a precision diamond saw, the MCFBs were trimmed to 1-cm longitudinal length and cross-sectional diameter of 1-mm. Polystyrene end caps of 1 mm length were bonded

to the ends of the MCFBs using a 1:1 acetone/toluene (v/v) mixture to anchor polystyrene fibers within the MCFB to an external, rigid material. Next, polystyrene side caps were bonded to the ends of MCFBs. The PMMA matrix was then selectively removed by immersing MCFB templates in 99.7% propylene carbonate (Sigma-Aldrich) at 45^{o}C for 24 hr; this was repeated 3 times to ensure complete removal of PMMA. The templates were then washed in 95% ethanol and distilled water prior to agarose casting.

In contrast to previous methods, various concentrations (0.2-14%w/w) of Ultrapure agarose (30 mg/mL; Sigma-Aldrich) were dissolved in distilled water at 100^{o}C. MCFB templates were kept wet in distilled water to reduce surface tension effects between the casted agarose and polystyrene mold. After cooling of agarose to 65^{o}C, wet MCFB templates were submerged into the agarose solution along the longitudinal axis, reducing perpendicular tension along 1cm fibers in the viscous solution. The template was slowly permeated through diffusion of the agarose solution into the water within the mold, as it was gently agitated at a 1mm amplitude every 5 seconds along the longitudinal axis only for 2 minutes. To maintain channel linearity, the agarose diffusion step is a necessary change to the previous published protocol when scaling the scaffold, reducing mold fracture of the sensitive polystyrene cable over longer distances, smaller cable sizes, and more closely packed cores. After maintenance at 65^{o}C for 5 min, the templates was removed from the agarose solution and placed into a glass well with a 100µL coating of agarose. Immediately after placing into the glass tray the template is covered in another layer of 100uL 65^{o}C

agarose. The thin layer sandwiching ensures complete permeation of the scaffold channels, while reducing dehydration and scaffold wall imperfections. Additionally, while cutting, the thin layer is more flexible and reduces fracture propagation imperfections common to larger and longer casts while scaling up. The thin layer of agarose was cut from the gelled agarose aggregate using a scalpel, leaving just an agarose scaffold cast, bound by polystyrene caps and fiber mold. The polystyrene fibers, end-caps, and side-caps were dissolved by immersing templates in 99% tetrahydrofuran (THF) (Sigma-Aldrich) at room temperature for 24 hr. This process was repeated 3 times to ensure complete removal of polystyrene, resulting in individual free-floating agarose scaffolds. Scaffolds were collected and washed in acetone, then stored in 95% ethanol for sterile storage until future use. One day before implantation the scaffolds were washed in 3 cycles of autoclaved distilled water and stored in a sterile UV hood until implantation into the conduit sleeve.

The *conduit sleeve* was made using Tygon Tubing with an inner diameter of 1.2mm and an outer diameter of 1.5mm. To provide a stable means to attach the peripheral nerve epineurium to the conduit sleeve, the conduit sleeve (Tygon Tubing) was cut with an additional 2mm beyond the length of the linearized agarose core. Upon implantation, the epineurium was sutured or glued to the conduit sleeve, avoiding direct attachment to the scaffold/host interface, which may cause channel destruction, channel obstruction, or mechanical inflammation (Fig. 4.2). Additionally, the conduit sleeve provides mechanical strength to the brittle agarose core of the implant upon movement, displaces tension from the sensitive tissue/scaffold interface,

and provides a simple means of delivery, storage and handling. The conduit sleeve was cut longitudinally with a scalpel and temporarily creased open for the implantation of the core component. After implantation of the linearized agarose core, the conduit sleeve was allowed to reformed back to the original shape (Fig. S1).

Analysis of scaffold structure

Scaffold microstructure was visualized under phase-contrast microscopic optics, as previously described (Stokols et al., 2006). Pore dimensions and porosity were directly measured from light micrographs. Templated agarose scaffolds with channel diameters of 200μm and a uniform wall thickness of 100μm were consistently produced over distances of 1cm. Additionally, the 46um wall and 278um channel scaffolds were consistently produced (Fig. S2). Scaffolds contained uniaxial channels organized in an array precisely replicating the pattern of polystyrene fiber cores in the MCFB. Scaffold microstructure was also examined under light level microscopy, and the rare scaffold with imperfections of channel architecture was not used for *in vivo* studies. Furthermore, to reduce drying and potential fracture of scaffolds, channel linearity was tested with a non toxic and non destructible test using air/water surface tension. The scaffolds were placed in a highly aerated room temperature water solution to trap micron sized bubbles within the channels. Under a light level microscope, the architecture could be visualized by the refraction of the air within the channels. Bubbles were removed by lowering the surface tension with alcohol washes, and the scaffold was washed in sterile water and ready for implantation after

inspection.

Superheating of agarose solution

A 25ml glass bottle with a highly sealable lid was tared and filled with 10 ml of distilled water. The desired amount of agarose was weighted out and poured into the water, the lid was tightly secured and the container the tare weight was taken again. The solution was then placed into a microwave on high setting for 10 seconds and agitated. The bottle was repeatedly placed into the microwave for 2 second intervals and carefully stirred for 2 seconds in between intervals, being careful not to cause a pressure spike with agitation. As more agarose was added the vapor pressure increased, permitting a higher boiling point. The seal on the lid was carefully observed, as to make sure it was not broken. The microwave increased the solution temperature, creating an increase in pressure within the sealed container, which increased the possible boiling point, resulting in a superheated solution. Therefore, using our superheating method, the solution temperature could reach much higher levels in the microwave, increasing the agarose solubility, and thus allowing for much greater agarose concentrations in the gel. To ensure no changes in concentrations, the final tare weight was compared to the initial tare weight of the container. The incremental changes in agarose concentration were intended to increase the resulting strength of the agarose gel.

Measurement of agarose relative fracture strength

To measure the relative fracture strength of the agarose gel at different concentrations, a basic failure test was applied. Over various concentrations ranging from 0.2% to 14%, an agarose gel was pored into a 5ml plastic well and let dry after superheating for 1 hr. Using a #2 scalpel, six blocks of $4mm^3$ size were cut for each concentration. The blocks were placed on a Kimwipe to reduce sliding of the sample under compression. A 3 inch plastic cell culture dish was placed on top of the sample and adjusted to be flush with the surface of the bench. A ruler was used to ensure the dish was centered and flush with the desktop. A glass flask was vertically stabilized with three heavy glass cylinders as external guides, and placed on top of the sample. Immediately weight was added at a rate of 50g/sec until visible fracture of the sample. Samples were not pre-loaded. The data was collected and averaged over six samples for each concentration. The change in fracture strength was fit to a curve with a R^2 of 0.95 or greater. The fracture strengths were normalized to the 1% agarose concentration to normalize the surface area. Thus the relative fracture strength was scalable to our agarose scaffold design. All concentrations were compared using ANOVA and post-hoc fisher t-tests.

Modeling growth factor release from agarose

Bovine Serum Albumin was used to model growth factor release from the agarose, because of its similar Stokes radius(4nm) to BDNF, NGF, and NT-3. A 2mm thick section of agarose was created on to bottom of a 2cm diameter glass bottle. A

4% agarose solution was used. BSA was dissolved in distilled water at 5 and 10% solutions. Two groups were created (n=6) by applying the two different BSA concentrations to the 4% agarose. Two weeks were allowed for equilibrium to be reached with a 10ml BSA wash in the agarose gel containers. After removing the BSA solutions the diffusion test was conducted. One ml of fresh distilled water was added to the agarose gel container and collected every hour. The media was lyophilized and weighed. The amount of BSA released was a function of the remaining weight within the lyophilized media. The release profile was measured and compared between groups to determine if the release was diffusion or equilibrium mediated.

Peripheral nerve lesion surgery and scaffold implantation

Adult female Fischer 344 rats (n=12) weighing 150-200 g were used. Institutional guidelines on animal care were strictly followed. Under deep anesthesia, the sciatic nerve was exposed at mid-thigh and cut with microscissors to the desired lesion length, 2mm (n=3), 5mm (n=6), and 10mm (n=3) segments of sciatic nerve were removed. The sleeve of the conduit was sutured (10-0 polyester) or glued (cyano-methacrylate) to the sciatic nerve ends along the epineurium, while ensuring the nerve stumps were in direct contact with the agarose core inside the conduit. Nine animals were sacrificed after 4 weeks (recipients of 2mm, 5mm, and 10mm conduits) and three (recipients of 5mm conduits) after one year. Scaffolds were empty upon implantation.

Immunohistochemical labeling

Animals were transcardially perfused with 100ml of cold phosphate buffered saline (PBS), followed by 300 ml of 4% paraformaldehyde in phosphate buffer. Sciatic nerves were removed, postfixed overnight in 4% paraformaldehyde, and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose at 4°C. Tygon tubing was removed and sciatic nerves were blocked in TBS Tissue Freezing Media (Triangle Biomedical Sciences) on dry ice, and sagittal or coronal sections were cut on a freezing microtome set at 35 µm intervals.

Sciatic nerve sections were labeled for neurofilament (NF-200kD, 1:1000, Chemicon). Free-floating or direct mounted sections were placed in TBS-Triton 0.25% for 3x10 minutes and then in a serum blocking solution of 4% donkey serum with TBS-Triton 0.25% for 3 hrs. Next, they were washed in TBS for 3x10 minutes and placed in primary antibody solution overnight. The next day, sections were washed for 3x10 minutes and placed in a secondary antibody for 5 hrs (6:1000 Chemicon 488 and 594 anti- goat, mouse, or rabbit, respectively). Additionally, ribosomal RNA (Nissl, cresyl violet acetate, Polysciences, Inc.) was stained to examine general cellular morphology.

Results

The linearized agarose scaffold core can be scaled up to 1cm length and retain linearity over this distance

To achieve uniform scaffolds over a distance of 1cm, we modified our previously published protocol for scaffold manufacturing by using methods to reduce surface tension and increase agarose diffusion. Templated agarose scaffolds were produced with 2 different cross sectional lumina over 1cm length: 1) channel diameters of 200 μm with a wall thickness of 100 μm, providing a lumen cross sectional area of 45%. 2) 278μm channel diameter and 46μm wall thickness, providing a lumen cross section area of 74%. The hydrogel scaffolds were soft and flexible, qualitatively resembling normal peripheral nerve elasticity. Scaffolds contained uniaxial channels organized in an array precisely replicating the pattern of polystyrene fiber cores in the MCFB. Serial section microscopic analysis of scaffolds subjected to *in vitro* testing indicated that channel structure remained consistent throughout the full length of the scaffolds, without evidence of wall breakdown or core coalescence in sections sampled every 1 mm along the 10-mm length of scaffolds (Fig. 4.3A). The mean cross-sectional diameters of the two scaffolds were 200±10 μm and 280±10 μm channel diameter and mean wall thickness were 100±10 mm and 44±10, respectively. The lumen cross sectional area of each scaffold available for axonal growth was 44±8% and 76±8%. *In vivo* testing indicated that the scaffolds did

not appear to undergo degradation from hydrolysis over a 1-year testing period (Fig. 4.3).

Superheating with high agarose gel concentrations increases scaffold strength

To reduce potential for channel collapse caused from subject movement after implantation, it may be necessary to increase the agarose strength of the scaffold. The strength of agarose gel was increased using superheating to permit a larger percentage of agarose in solution before casting the templated scaffold. The fracture strength increased as the percentage of agarose concentration increased to 14%. The fracture strength of the gel was normalized to a 1% gel and fit to a nearly linear curve of y = -111006x2 + 30334x - 29.197 (Chi squared = 0.9867) (Fig. 4.4). When comparing 1% to 14% (w/w) agarose concentration, the gel fracture strength was increased by an order of magnitude (ANOVA, $p<0.05$) (Fig. 4.4). The effects of increasing agarose concentration and other scaffold parameters were modeled by applying Euler's buckling equation (Fig. S3,S4).

Scaffold growth factor release controlled by surface effects and not internal resistance

Schwann cells in nerve autografts release growth factors, which promote survival axonal growth and guidance in nerve regeneration. Therefore, a nerve conduit, which can release a transient growth factors gradient, such as NT-3, BDNF, or NGF, may enhance axonal survival and guidance. Growth factor release was

modeled using BSA because of its highly similar stokes radius of 4nm compared to growth factors. Based on the agarose average pore sizes of about 40nm with most concentrations (Narayanan et al., 2006), and the stokes radius of BSA (approximately 4nm), it is expected that the BSA released from the scaffold is limited by equilibrium with water and not diffusion from the agarose. Two different BSA concentrations of 5% and 10% were released from an agarose plug. The release of BSA from the gel was measured with each hour by lyophilizing the media changes. As expected, the BSA release profiles were dramatically reduced with the first media change, indicating a large amount of BSA release within the first hour, likely limited by equilibrium. Approximately 94% of the BSA was released within 4 media changes. Furthermore, when considering the two concentrations, the gels released exactly double the amounts of material with successive media change, illustrating the concentration profile mediated by solution equilibrium within the time scale of one hour (and not Fick's law) (Fig. 4.5). Thus, the limiting factor in growth factor release profile was dictated by the surrounding media. Therefore, when implanted into an injury site, the diffusion will likely be limited by the boundary layer conditions along the scaffold/host interface, created from the fluid/tissue in the biological system.

Conduit is biocompatible after one year, supporting cellular migration and axonal extension into the channels

A peripheral nerve conduit was inserted into a 5mm sciatic nerve lesion to test for biocompatibility and axon permissiveness across the channels of the linearized

agarose scaffold core. Inspection of injury sites revealed that the peripheral nerve gap was completely filled with the implant, the proximal and distal surface of the scaffold was cohesive with the distal and proximal ends of the peripheral nerve, and there was no fibrotic degeneration or scaffold extrusion (Fig. 4.6). At the microscopic level, individual, uniaxial agarose walls were clearly visible in Nissl-stained sagittal sections (Fig. 4.6 B). To confirm that the synthesized honeycomb arrangement of channels remained intact one year after in vivo placement, two conduits were sectioned coronally. In both cases, Nissl-stained sections revealed a honeycomb structure, identical to the initial structure of the scaffold prior to implantation (Fig. 4.3B).

The biocompatibility of the scaffold was assessed by analyzing cellular responses within, and at the borders of the scaffolds. Nissl-stained sections confirmed that fibrous tissue encapsulation of scaffolds did not occur. Host cells did not penetrate through agarose walls of the scaffold, instead they penetrated scaffolds through either open aperture that was opposed to the transected nerve stump. Host cell migration and neurofilament axon extension into the channels was clearly evident (Fig. 4.6). Additionally, conduit implants became vascularized, as revealed by macro light level imaging, while blood vessels clearly aligned along the longitudinal orientation of the channels (Fig. 4.7). After one month, neurofilament axons extended through the entire length of 2mm (n=3) and 5mm (n=3) conduits while maintaining a qualitatively linear profile within the channels. Only one of three 10mm-long scaffolds contained neurofilament axons at the distal end of the scaffold, however. Additionally, after one year, neurofilament axons extended through the entire length of 5mm (n=3) conduits.

Total axon linearity, density, and penetration into the sciatic nerve *beyond* the lesion site were not quantified and will be the subject of future study.

Discussion

In the PNS, the current challenges of nerve regeneration research are to improve axonal growth beyond gaps of 1cm in length, thereby augmenting functional recovery. If successful, these approaches would eliminate the need for sural nerve autografts which are associated with patient morbidity. In the present study we demonstrate fabrication of linearized agarose conduits over lengths of 2mm to 10mm. Furthermore, the design is scalable to longer lesion distances, lumen cross sectional areas, and fracture strengths. Using methods to reduce surface tension and increase agarose diffusion, the scaffold was scaled from 2mm to 1cm lengths. By adjusting the size of the extruded polymers in the template, the lumen cross sectional area was changed from 45% to 74%. The agarose gel concentrations were increased from 0.2% to 14% through novel superheating methods, scaling the fracture strength by an order of magnitude. Additionally, we determined that the rate of BSA (similar stokes radius to growth factors) release from the agarose was determined by unimpeded release into the surrounding medium, and was not limited by binding to agarose or constrained by the pore size of agarose walls (approximately 50nm, Narayanan et al., 2006). Finally, these scaffolds are biocompatible after 1 year while supporting axon growth to the

disal aspect of a 5mm length scaffold.

In our conduit design, our aim was to mimic key characteristics of an autologous nerve graft. The linearity within the channels mimics the innate guiding architecture found within the endoneurial tubes (collegen fibrils) of a nerve autograft (Ushiki and Ide, 1990). The scaffold channels have the potential to be filled with an axon permissive extracellular matrix or cellular substrate to mimic the native cellular milieu in the nerve autograft. Additionally, the scaffold channel and wall sizes can be scaled to adjust the effective lesion volume, potentially enhancing the rate at which the empty lesion cavity is filled with a substrate, such as fibrin, that is permissive to axon growth. The fracture strength of the scaffold can be designed to simulate the mechanical properties of the nerve or be enhanced to provide a sturdy conduit, both of which may protect the sensitive interface between the scaffold and nerve tissue. Additionally, the BSA release study illustrated that the scaffold/host interface will dictate growth factor release.

Several natural and synthetic conduits have been tested to date to promote peripheral nerve regeneration (Hudson et al., 2000; Wang et al., 1993). However, none successfully address precise microstructure linearity within a biocompatible hydrogel (Dooblah, et al., 1996; Evans et al., 2001; Hudson et al., 2000; Aldini et al., 1996; Madison et al., 1985; den Dennen et al., 1996; Langone et al., 1995; Lundborg and Hansson, 1980; Molander et al., 1982; Chiu and Strauch, 1990). Thus, our scaffold design has the advantage of generating consistent linear channels that are hypothetically capable of retaining the natural organization of the intact nerve to guide

axons to their pre-injured targets. Non-linear channels suffer from the drawback that, as lesion length increases, progressively greater numbers of axons may become misaligned with the original targets, reducing the possibility of functional restoration.

A potential disadvantage of agarose as an scaffold is its lack of biodegradability. It is commonly thought that a scaffold implant should degrade after implantation to permit complete host tissue integration at the lesion site (Schmidt, 2003). A biodegradable scaffold will provide a more mechanically stable transition between the injury site and the reinnervated nerve. Thus, manufacture of a templated biodegradable scaffold, such an alginate hydrogel, is being explored.

Growth factor delivery from a scaffold may potentially enhance neuronal survival and promote axon extension (Evans., 2001; Terenghi, 1999; Babensee, 2000). Growth factors have been delivered using osmotic pumps or direct injections to the nerve, which may not be suitable for clinical application (Babensee, 2000). Thus, a scaffold that can serve as a source of growth factors would be advantageous. A scaffold with similar pore size to the protein Stokes radius would cause internal diffusion limitations within a medium. Due to large pore sizes, many biocompatible hydrogels will not retain molecules the size of growth factors (Hoffman, 2002; Peppas et al., 2000; Lee and Mooney, 2001). This is an undesirable feature to enhance axon regeneration over long durations and distances as it will inevitably result in a burst release of growth factors mediated by the host scaffold interface. Ideally, a scaffold with strong internal diffusion properties, would transiently release bioactive growth factors in a concentration capable of promoting axon growth, over a duration in which

axon support is needed (weeks). While our conduit design is capable of containing growth factors within the agarose walls, the release is directly mediated by the host environment, and not controllable due to the large pore sizes. However, if an osmotic pump is attached to the conduit in a removable, non-invasive manner, growth factors could be readily and transiently dispersed through the agarose walls to regenerating axons. The uniform honeycomb structure, and ease of growth factor diffusion through the agarose, provide an ideal means to,uniformly deliver growth factors along the scaffold.

Future studies can take advantage of the high degree of "scalability" of the nerve conduit to further optimize its properties for axonal growth: 1) The effective lumen cross sectional area can be modified depending on the lesion volume to reduce time to fill the empty cavity. In theory, by reducing the time needed to fill the empty lesion cavity, the axons can bridge the lesion sooner, potentially reconnecting to their target before they become unviable. 2) The mechanical interface between the cellular tissue and the hydrogel is a critical issue in biomaterial design. Basic structural theory suggests that mimicking the elasticity of the nerve would reduce separation failures upon movement of the interface. However, weaker lumen may collapse over long-term implantation, therefore a stiffer, more rigid biomaterial may prove useful. Additionally, the conduit sleeve may alleviate stresses along the host interface and protect the lumen channels from collapsing. Therefore, depending on the strength of the conduit sleeve, the appropriate agarose strength will likely need to be optimized for each conduit system. 3) The inclusion of substrates into the lumen channels might

enhance axon growth, Scwhann cells, laminin, or collagen (Hadlock et al., 2000; Matsumoto et al., 2000; Li et al., 1992) might be suitable candidates. A substrate that enhances axon growth and does not degrade under storage conditions would be ideal. 4) As discussed above, transient growth factor delivery may be feasible through the use of an osmotic minipump (i.e. Alzet). An osmotic pump can be connected to the conduit sleeve in a removable and non-invasive manner. Different growth factors, release rates, and durations need to be tested to maximize axon growth. 5) A biodegradable conduit core might also benefit the design (discussed above). Specifically, a hydrogel such as alginate, would be adaptable to our current casting process.

Thus, by mimicking a nerve autograft with scalable design parameters, this conduit design provides the basis for future studies aiming to increase the number of regenerating axons, the speed of axon regeneration, and functional outcomes after peripheral nerve injuries.

Acknowledgement

Chapter 4 is original work in preparation as Gros TR, Sakamoto J, Tuszynsksi MH. A highly linearized and mechanically scalable hydrogel nerve conduit for peripheral nerve regeneration and is included with permission from all the manuscript's authors. The dissertation author was the primary author of this paper.

References

Arai, T, G Lundborg and L Dahlin (2000) Bioartificial nerve graft for bridging extended nerve defects in rat sciatic nerve based on resorbable guiding filaments. Scand J Plast Reconstr Hand Surg 34: 101-08.

Archibald, S, C Krarup, J Shefner, S Li and R Madison (1991) A collagen-based nerve guide conduit for peripheral nerve repair: An electrophysiological study of nerve regeneration in rodents and nonhuman primates. The Journal of Comparative Neurology 306(4): 685-96.

Archibald, S, J Shefner, C Krarup and R Madison (1995) Monkey median nerve repaired by nerve graft or collagen nerve guide tube. Journal of Neuroscience 15(5): 4109-23.

Babensee, J, L Mcintire and A Mikos (2000) Growth factor delivery for tissue engineering. Pharmaceutical Research 17(5): 497-504.

Britland, S, P Clark, P Connolly and G Moores (1992) Micropatterned substratum adhesiveness: A model for morphogenetic cues controlling cell behavior. Experimental cell research 198(1): 124-29.

Cai, J, X Peng, K Nelson, R Eberhart and G Smith (2004) Synergistic improvements in cell and axonal migration across sciatic nerve lesion gaps using bioresorbable filaments and heregulin-beta1. Journal of Biomedical Materials Research Part A 69(2): 247-58.

Chiu, D (1995) Special article: The development of autogenous venous nerve conduit as a clinical entity. P & S Medical Review 3(1).

Clark, P (1992) Cell guidance by micropatterned adhesiveness in vitro. Journal of Cell Science 103(1): 287-92.

Clark, P (1993) Growth cone guidance and neuron morphology on micropatterned laminin surfaces. Journal of Cell Science 105(1): 203-12.

Clark, P (1993) Growth cone guidance and neuron morphology on micropatterned laminin surfaces. Journal of Cell Science 105(1): 203-12.

Doolabh, V, M Hertl and S Mackinnon (1996) The role of conduits in nerve repair: A review. Rev Neurosci 7(1): 47-84.

Evans, G, K Brandt, A Niederbichler, P Chauvin, S Hermann, M Bogle, L Otta, B Wang and C Patrick Jr (2003) Clinical long-term in vivo evaluation of poly (l-lactic acid) porous conduits for peripheral nerve regeneration.

Evans, G, K Brandt, M Widmer, L Lu, R Meszlenyi, P Gupta, A Mikos, J Hodges, J Williams and A Gürlek (1999) In vivo evaluation of poly (l-lactic acid) porous conduits for peripheral nerve regeneration. Biomaterials 20(12): 1109-15.

Evans, G (2001) Peripheral nerve injury: A review and approach to tissue engineered constructs. The Anatomical Record 263(4): 396-404.

Evans, G, K Brandt, S Katz, P Chauvin, L Otto, M Bogle, B Wang, R Meszlenyi, L Lu and A Mikos (2002) Bioactive poly (l-lactic acid) conduits seeded with schwann cells for peripheral nerve regeneration. Biomaterials 23(3): 841-48.

Hadlock, T, J Elisseeff, R Langer, J Vacanti and M Cheney (1998) A tissue-engineered conduit for peripheral nerve repair. Archives of Otolaryngology-Head and Neck Surgery 124(10): 1081-86.

Hadlock, T, C Sundback, D Hunter, M Cheney and J Vacanti (2000) A polymer foam conduit seeded with schwann cells promotes guided peripheral nerve regeneration. Tissue Engineering 6(2): 119-27.

Haftek, J and P Warsaw (1970) Stretch injury of peripheral nerve. Journal of Bone & Joint Surgery, British Volume.

Haftek, J and P Thomas (1968) Electron-microscope observations on the effects of localized crush injuries on the connective tissues of peripheral nerve. Journal of Anatomy 103(Pt 2): 233.

Hammarback, J, J Mccarthy, S Palm, L Furcht and P Letourneau (1988) Growth cone guidance by substrate-bound laminin pathways is correlated with neuron-to-pathway adhesivity. Developmental biology(Print) 126(1): 29-39.

Hoffman, A (2002) Hydrogels for biomedical applications. Advanced Drug Delivery Reviews 54(1): 3-12.

Hollander, A and P Hatton (2004). Biopolymer methods in tissue engineering, Humana Press.

Hsu, S, C Chang, C Tang and F Lin (2004) In vitro and in vivo effects of ginkgo biloba extract egb 761 on seeded schwann cells within poly (dl-lactic acid-co-glycolic acid) conduits for peripheral nerve regeneration. Journal of Biomaterials Applications 19(2): 163.

Hudson, T, G Evans and C Schmidt (2000) Engineering strategies for peripheral nerve repair. Orthopedic Clinics of North America 31(3): 485-97.

Kam, L, W Shain, J Turner and R Bizios (2001) Axonal outgrowth of hippocampal neurons on micro-scale networks of polylysine-conjugated laminin. Biomaterials 22(10): 1049-54.

Kelsey, J, A Praemer, L Nelson, A Felberg and D Rice (1997). Upper extremity disorders: Frequency, impact, and cost, Churchill Livingstone.

Khademhosseini, A, R Langer, J Borenstein and J Vacanti (2006) Microscale technologies for tissue engineering and biology. Proceedings of the National Academy of Sciences 103(8): 2480-87.

Kleinfeld, D, K Kahler and P Hockberger (1988) Controlled outgrowth of dissociated neurons on patterned substrates. Journal of Neuroscience 8(11): 4098-120.

Lee, K and D Mooney (2001) Hydrogels for tissue engineering. CHEMICAL REVIEWS 101(7): 1869-80.

Li, S, S Archibald, C Krarup and R Madison (1992) Peripheral nerve repair with collagen conduits. Clinical materials 9(3-4): 195-200.

Lundborg, G (1988). Nerve injury and repair, Churchill Livingstone New York.

Lundborg, G, L Dahlin and N Danielsen (1991) Ulnar nerve repair by the silicone chamber technique. Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery 25(1): 79-82.

Lundborg, G, L Dahlin, D Dohi, M Kanje and N Terada (1997) A new type of “bioartificial” nerve graft for bridging extended defects in nerves. Journal of Hand Surgery 22(3): 299-303.

Lundborg, G (2003) Nerve injury and repair-a challenge to the plastic brain. Journal of the Peripheral Nervous System 8(4): 209-26.

Ma, Z, M Kotaki, R Inai and S Ramakrishna (2005) Potential of nanofiber matrix as tissue-engineering scaffolds. Tissue Engineering 11(1-2): 101-09.

Mackinnon, S and A Dellon (1988). Surgery of the peripheral nerve, Thieme Medical Publishers.

Matsumoto, K, K Ohnishi, T Kiyotani, T Sekine, H Ueda, T Nakamura, K Endo and Y Shimizu (2000) Peripheral nerve regeneration across an 80-mm gap bridged by a polyglycolic acid (pga)–collagen tube filled with laminin-coated collagen fibers: A histological and electrophysiological evaluation of regenerated nerves. Brain Research 868(2): 315-28.

Millesi, H (1967) Nerve transplantation for reconstruction of peripheral nerves injured by the use of the microsurgical technic. Minerva Chir 22(17): 950-1.

Millesi, H (1979) Microsurgery of peripheral nerves. World Journal of Surgery 3(1): 67-79.

Millesi, H (1990) Progress in peripheral nerve reconstruction. World Journal of Surgery 14(6): 733-47.

Narayanan, J, J Xiong and X Liu (2006). Determination of agarose gel pore size: Absorbance measurements vis a vis other techniques. Journal of Physics: Conference Series, Institute of Physics Publishing.

Ngo, T, P Waggoner, A Romero, K Nelson, R Eberhart and G Smith (2003) Poly (l-lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions. Journal of Neuroscience Research 72(2): 227-38.

Noble, J, C Munro, V Prasad and R Midha (1998) Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. The Journal of Trauma: Injury, Infection, and Critical Care 45(1): 116.

Norman, J and T Desai (2006) Methods for fabrication of nanoscale topography for tissue engineering scaffolds. Annals of Biomedical Engineering 34(1): 89-101.

Peppas, N, P Bures, W Leobandung and H Ichikawa (2000) Hydrogels in pharmaceutical formulations. European Journal of Pharmaceutics and Biopharmaceutics 50(1): 27-46.

Rajnicek, A (1997) Contact guidance of cns neurites on grooved quartz: Influence of groove dimensions, neuronal age and cell type. Journal of Cell Science 110(23): 2905-13.

Schmidt, C and J Leach (2003) N eural t issue e ngineering: Strategies for repair and regeneration. Annual Reviews in Biomedical Engineering 5(1): 293-347.

Sinis, N, H Schaller, C Schulte-Eversum, T Lanaras, B Schlosshauer, M Doser, K Dietz, H Rosner, H Muller and M Haerle (2007) Comparative neuro tissue engineering using different nerve guide implants. Axta Neurochirgica-Supplementum then Supplment-Wein 100: 61.

Spencer, P and A Lieberman (1971) Scanning electron microscopy of isolated peripheral nerve fibres. Normal surface structure and alterations proximal to neuromas. Z Zellforsch Mikrosk Anat 119(4): 534-51.

Stokols, S, J Sakamoto, C Breckon, T Holt, J Weiss and M Tuszynski (2006) Templated agarose scaffolds support linear axonal regeneration. Tissue Engineering 12(10): 2777-87.

Sunderland, S and J Smith (1969) Nerves and nerve injuries. Plastic and Reconstructive Surgery 44(6): 601.

Tai, H and H Buettner (1998) Neurite outgrowth and growth cone morphology on micropatterned surfaces. Biotechnology Progress 14(3): 364-70.

Taniuchi, M, H Clark and E Johnson (1986) Induction of nerve growth factor receptor in schwann cells after axotomy. Proceedings of the National Academy of Sciences 83(11): 4094-98.

Terenghi, G (1999) Peripheral nerve regeneration and neurotrophic factors. Journal of Anatomy 194(1): 1-14.

Ushiki, T and C Ide (1986) Three-dimensional architecture of the endoneurium with special reference to the collagen fibril arrangement in relation to nerve fibers. Arch Histol Jpn 49(5): 553-63.

Ushiki, T and C Ide (1990) Three-dimensional organization of the collagen fibrils in the rat sciatic nerve as revealed by transmission-and scanning electron microscopy. Cell and Tissue Research 260(1): 175-84.

Von Philipsborn, A, S Lang, A Bernard, J Loeschinger, C David, D Lehnert, M Bastmeyer and F Bonhoeffer (2006) Microcontact printing of axon guidance molecules for generation of graded patterns.

Xu, C, R Inai, M Kotaki and S Ramakrishna (2004) Aligned biodegradable nanofibrous structure: A potential scaffold for blood vessel engineering. Biomaterials 25(5): 877-86.

Yu, X and R Bellamkonda (2003) Tissue-engineered scaffolds are effective alternatives to autografts for bridging peripheral nerve gaps. Tissue Engineering 9(3): 421-30.

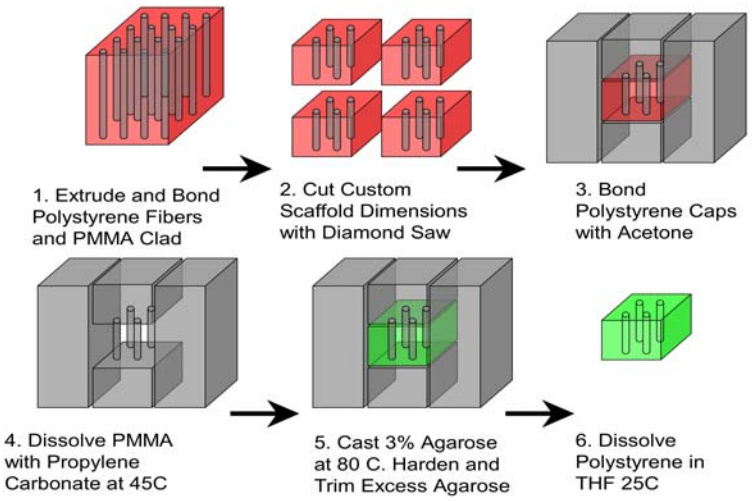

Fig. 4.1 Manufacturing of linearized agarose conduit. To develop a 1cm x 1mm x1mm core of the peripheral nerve conduit, we modified and our previously published methods to develop the linearized agarose scaffold (Stokols et al., 2006). Scaffolds were made using Multi-component fiber bundles (MCFBs) with various wall/channel ratios, resulting in different lumen cross sectional areas. Fibers were arranged in a hexagonal close-packed array separated by a continuous matrix of poly(methyl methacrylate) (PMMA). 1) MCFBs were simultaneously extruded and fused such that the polystyrene fibers were oriented parallel to the longitudinal axis of the bundles. 2) The MCFBs were trimmed to 1 cm longitudinal length and cross-sectional diameter of 1 mm. 3) The polystyrene fibers were anchored with polystyrene side and end caps using a 1:1 acetone/toluene (v/v) mixture. 4) Next, the mold was created by selectively removing the PMMA matrix by immersion in propylene carbonate, followed an alcohol and water wash. 5) Next, various concentrations (0.2-14%w/w) of Ultrapure agarose gel were created by superheating in the microwave for the casting step. The polystyrene mold was *kept wet* in distilled water to *reduce surface tension* effects between the casted agarose and polystyrene cables. The template was slowly permeated *through diffusion* of the agarose solution into the water within the mold, and gently agitated. After the casting step, the mold was sandwiched with thin films of agarose to ensure complete permeation, reduce dehydration, and *reduce fracture propagation* during mold separation step. The thin layer of agarose was cut from the gelled agarose aggregate using a scalpel, leaving just an agarose scaffold cast, bound by polystyrene caps and fiber mold. 6) The polystyrene fibers, end-caps, and side-caps were dissolved by immersing templates in tetrahydrofuran. Scaffolds were collected, washed in acetone, then stored in 95% *ethanol for sterile storage* before implantation into the conduit sleeve.

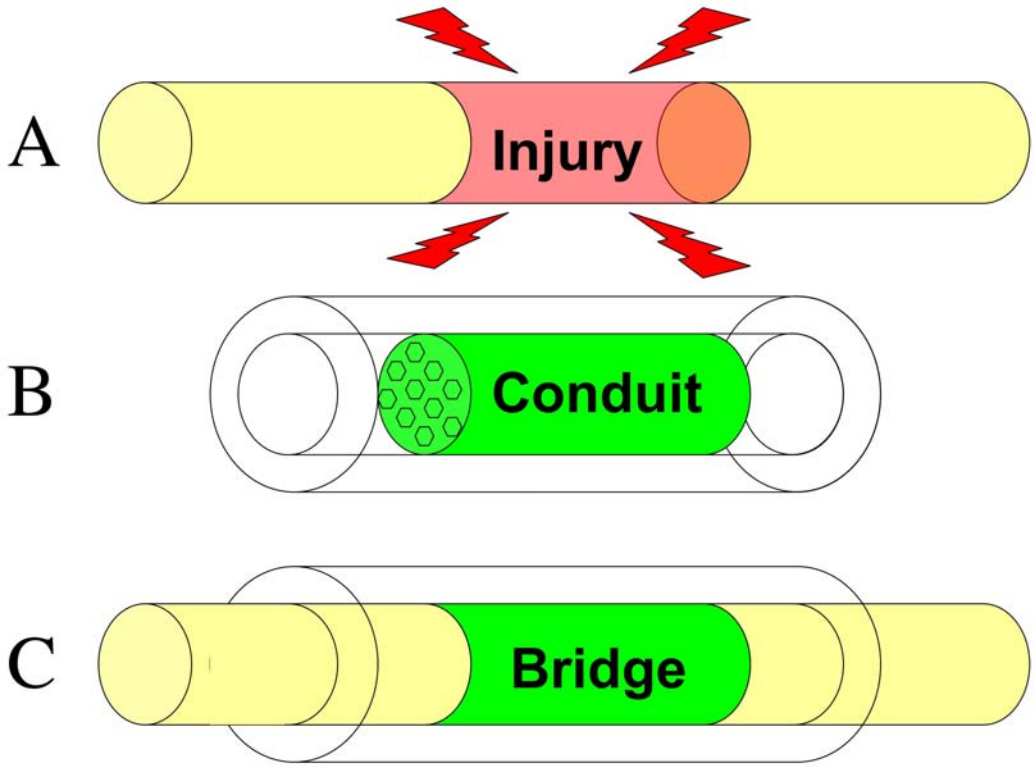

Fig. 4.2 The conduit bridges the peripheral nerve lesion gap. A) When a peripheral nerve lesion is greater than 4mm, a bridge is needed to prevent damage created from tension effects on the two nerve ends. B) The scaffold conduit has a linearized agarose core placed within a Tygon Tubing sleeve. The sleeve has an additional 2mm of tubing on each end to permit suturing. C) The nerve ends are inserted into the conduit sleeves and up against the agarose core. For stabilization, the epineurium is sutured or glued to the highly stable conduit sleeve. The conduit mimics the guidance properties of endoneurial tubes and oriented Schwann cells with linearized channels. Additionally, the conduit is capable of supporting permissive substrates within the lumen (i.e. cells, laminin, etc.). Theoretically, growth factors could be delivered transiently to the core in a controlled manner with an external attachment through the side of the sleeve, such as an Alzet pump.

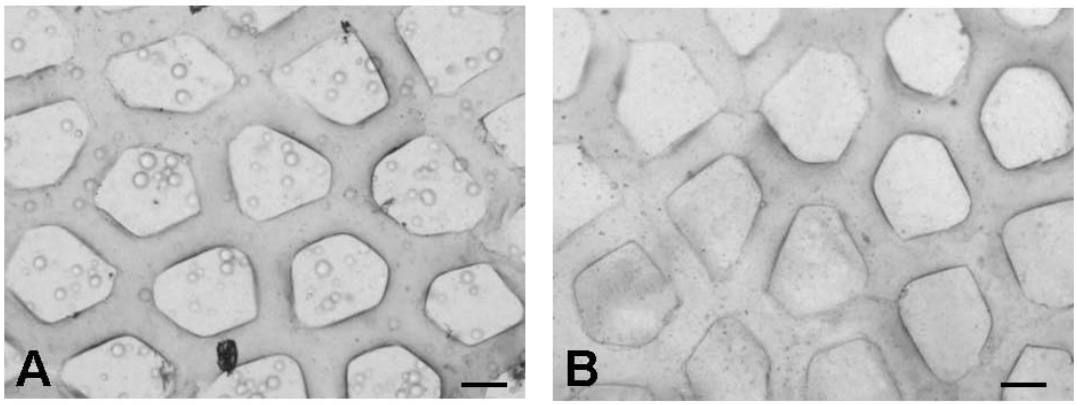

Fig. 4.3 Peripheral nerve scaffolds maintain structure one year post-implantation. A) Microscopic analysis of serial sections from scaffolds subjected to in vitro testing indicated that channel structure remained consistent through the full length of scaffolds. The mean cross-sectional diameters of the two scaffolds were 200±10 mm and 280±10 mm channel diameter and mean wall thickness were 100±10 mm and 44±10, respectively. The lumen cross sectional area of each scaffold available for axonal growth (scaffold volume) was 44±8 and 76±8 %. Furthermore, the scaffolds did not undergo mass degradation from hydrolysis over a 1-year testing period *in vitro*. B) To confirm that the synthesized honeycomb arrangement of channels remained intact one year after *in vivo* placement, two conduits were cut coronally. In both cases, Nissl-stained sections revealed a honeycomb structure, identical to the initial structure of the scaffold prior to implantation. Scale bars A),B)100um.

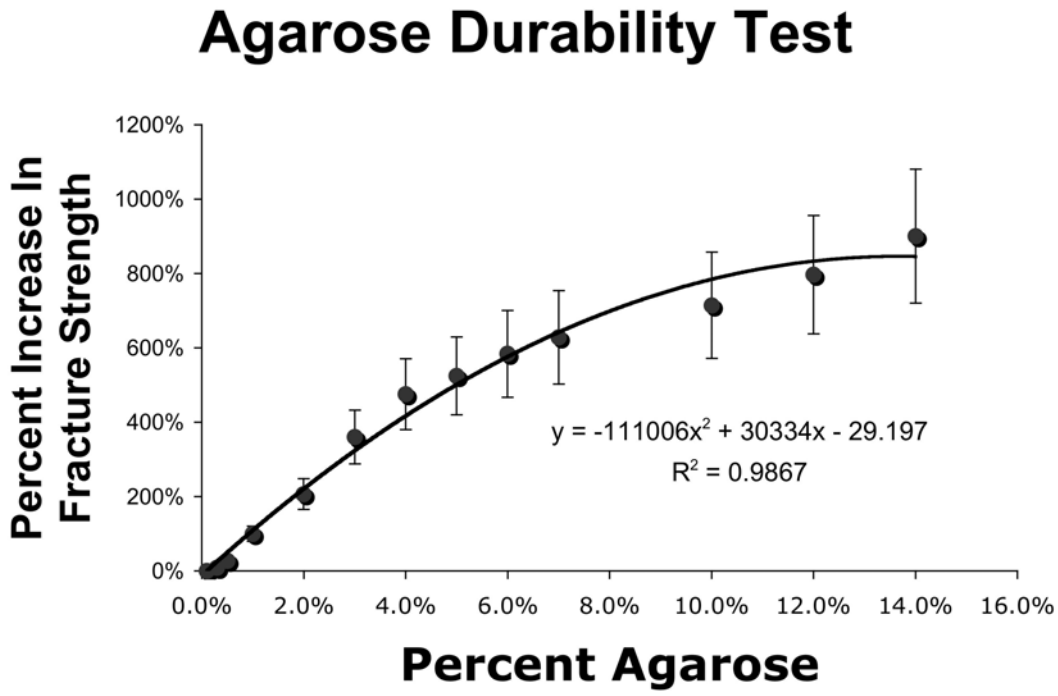

Fig. 4.4 The relative fracture strength of agarose gel was increased using a superheating method. The fracture strength increased as the percentage of agarose concentration increased up to 14%. The percent increase in fracture strengths are relative to 1% agarose. The normalized fracture strength of the gel strengths fit to a nearly linear curve of y = -111006x2 + 30334x - 29.197 (Chi squared = 0.9867). All groups were compared using ANOVA ($p<0.05$) a Fisher post hoc t-test. Specifically, when comparing 1% to 14% (w/w) agarose concentration, the relative fracture strength was increased by nearly an order of magnitude (9-fold) ($p<0.05$).

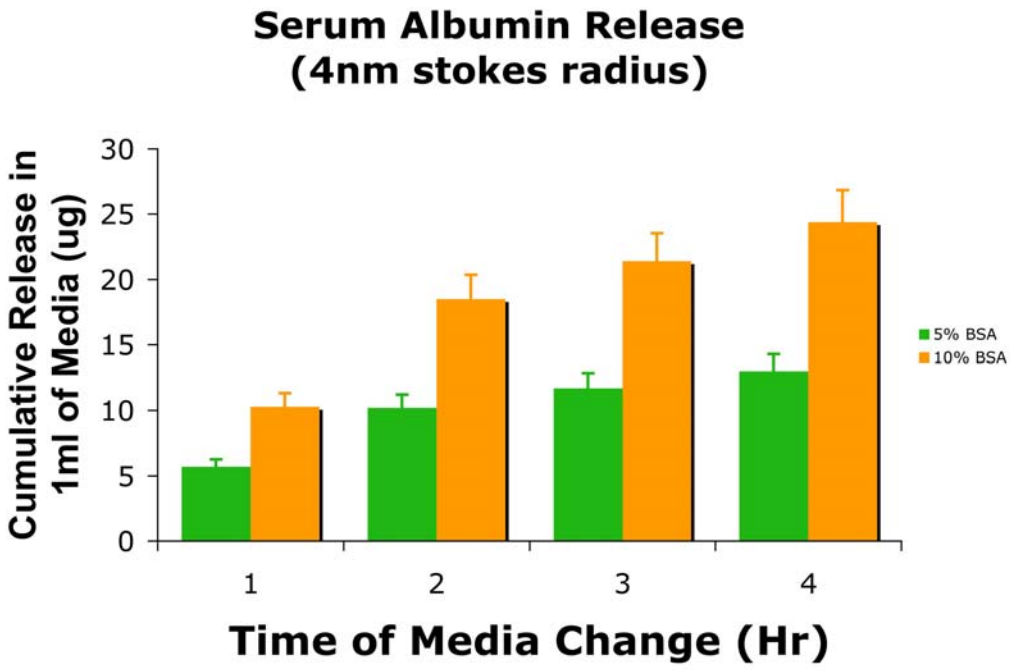

Fig. 4.5 A model for growth factor release from agarose. NT-3, BDNF, and NGF were modeled using BSA because of its similar stokes radius of 4nm. The BSA released from the scaffold was limited by equilibrium with water and not diffusion from the agarose. Two different BSA concentrations of 5% and 10% were released from an agarose plug. The release of BSA from the gel was measured hourly by lyophilizing the media changes. As expected, the BSA release profile was dramatically reduced after the first media change, indicating a large amount of BSA release within the first hour, likely limited by equilibrium. Approximately 94% of the BSA was released within 4 media changes. Furthermore, when considering the two concentrations, the gels released exactly double the amounts of material with successive media change, illustrating that the concentration was mediated by equilibrium within the one hour time scale (not Fick's law). Thus, the limiting factor in growth factor release profile is dictated by the surrounding media. Therefore, when implanted into an injury site, the diffusion will be limited by the boundary layer conditions along the scaffold/host interface, created from the fluid/tissue in the biological system.

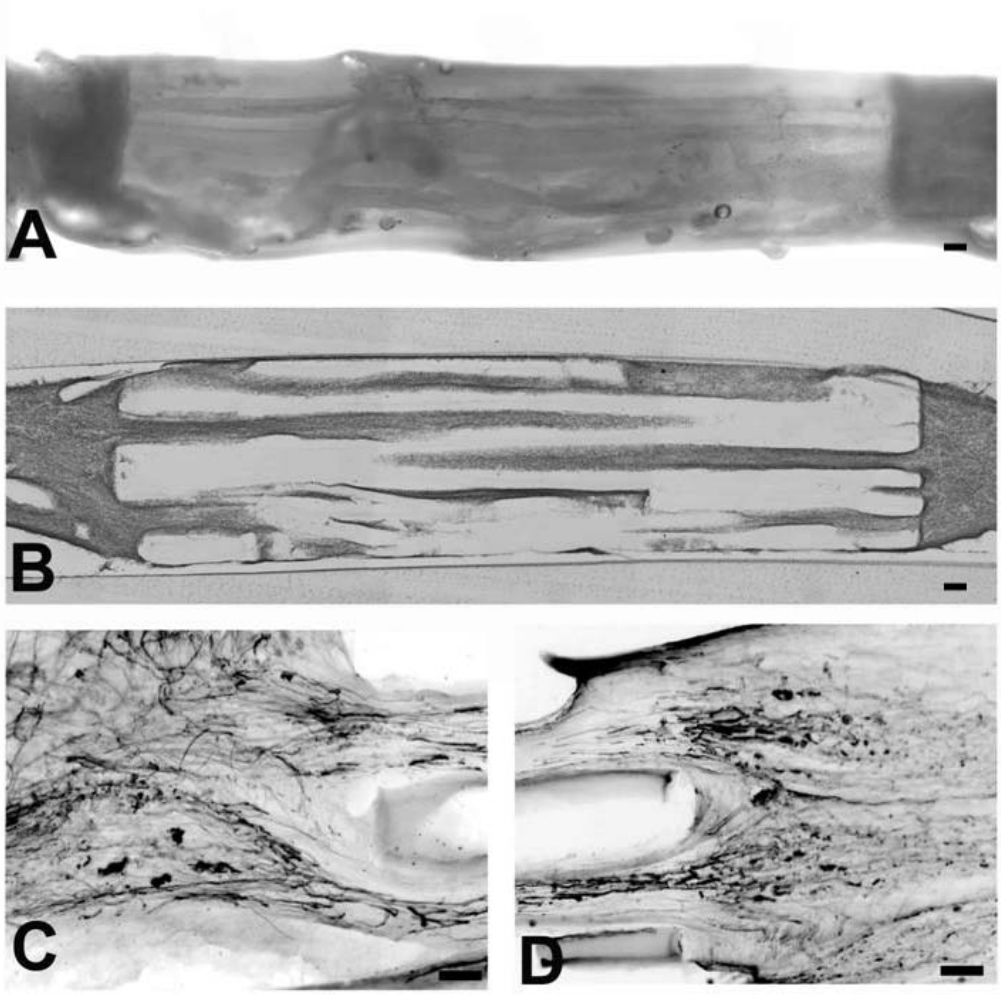

Fig. 4.6 Peripheral nerve conduits (n=3) contain nongranularized tissue 1 year following implantation and support axon growth over a length of 5mm .A) Inspection of injury site confirmed the peripheral nerve gap was completely filled with the implant and that scaffold ends were cohesive with the distal and proximal ends of the peripheral nerve. There was no fibrotic degeneration or scaffold extrusion. B) At the microscopic level, individual, uniaxial agarose walls were clearly visible in Nissl-stained sagittal sections. Nissl-stained sections confirmed that fibrous tissue encapsulation of scaffolds did not occur. Host cell migration into the channels was clearly evident, presumably from the luminal openings at either end of the scaffold. (C, D) Neurofilament labeling demonstrates axon growth into (C) the proximal end of the conduit and (D) axons exiting the scaffold at the distal end.. Scale bars A,B 200*u*m; C,D 100*u*m

Fig. 4.7 The linearized agarose conduit supported vascular penetration. As revealed by macro light level imaging, blood vessels were present in the conduit, traveling within the channels in a relatively linear manner. Scale bar 100*u*m.

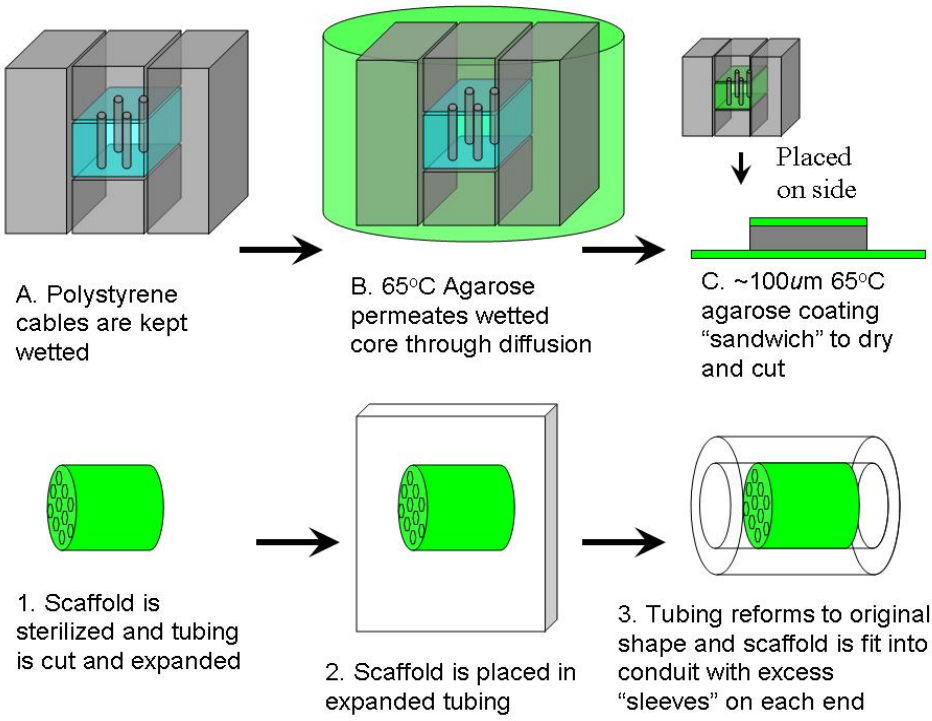

Fig. 4.S1 Modifications to templated agarose scaffold production. Changes in the casting procedure were necessary to scale up agarose scaffold to a one centimeter nerve conduit. MCFB templates were *kept wet* in distilled water to *reduce surface tension* effects between the casted agarose and polystyrene mold. After cooling of agarose to 65°C, wet MCFB templates were submerged into the agarose solution along the longitudinal axis, reducing perpendicular tension along 1cm fibers in the viscous solution. The template was slowly permeated *through diffusion* of the agarose solution. After maintenance at 65°C for 5 min, the templates was removed from the agarose solution and placed into a glass well with a 100µL *coating* of agarose. Immediately after placing into the glass tray the template is covered in *another layer* of 100uL 65°C agarose. The thin layer sandwiching ensures complete permeation of the scaffold channels, while reducing dehydration and scaffold wall imperfections. The scaffold edges can be shaped into a cylinder using a scalpel. To provide a stable means to attach the peripheral nerve epineurium to the conduit sleeve, the conduit sleeve (Tygon Tubing) was cut with an additional 2mm beyond the length of the linearized agarose core. The conduit sleeve was cut longitudinally with a scalpel and temporarily creased open for the implantation of the core component. After implantation of the linearized agarose core, the conduit sleeve was allowed to reformed back to the original shape.

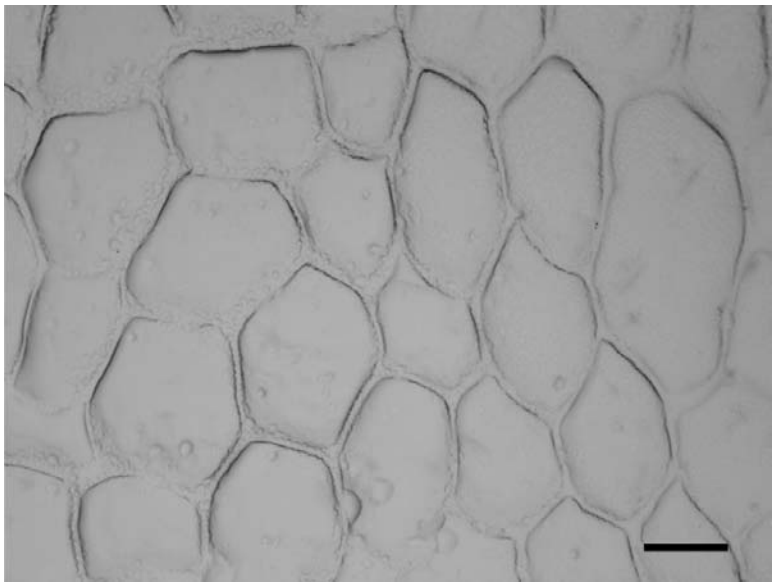

Fig. 4.S2 Scaffold of a lumen cross sectional area of 74%. The wall thickness was 46*u*m and the channel diameter was 278*u*m. Some structural damage occurred during the dehydration of the section onto the slide. Scale bar is 200*u*m.

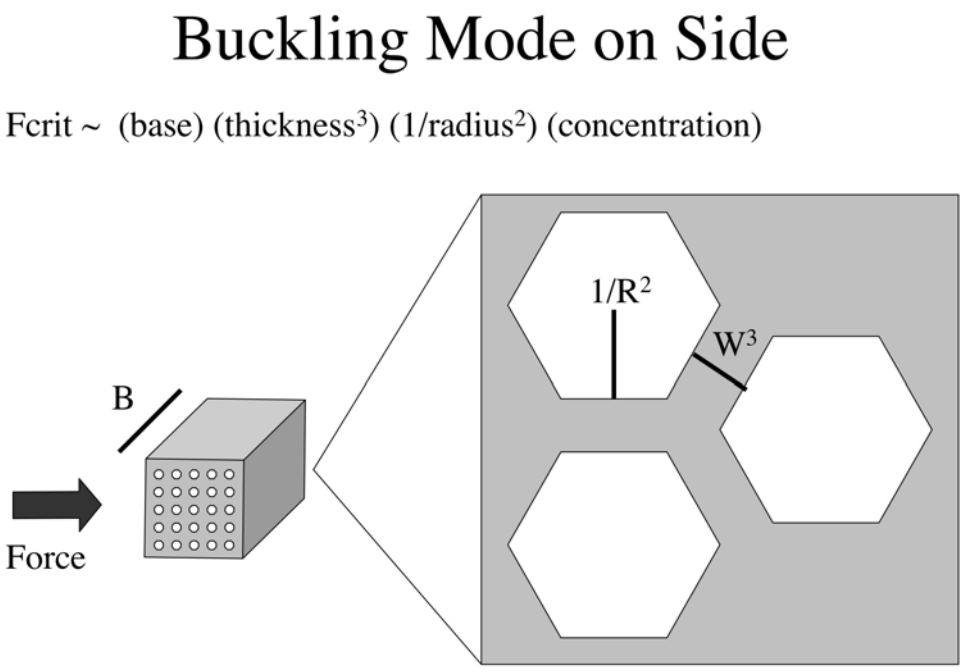

Fig. 4.S3 Buckling mode from the scaffold side. A) A modification of Euler's buckling is used to model potential methods to strengthen the scaffold design. An increase in channel radius quickly reduces the scaffold strength. Increasing the wall thickness will most quickly strengthen the scaffold. Near the critical fracture point, the elasticity modulus is approximated as a function of the fracture strength and concentration profile. Thus, Fcrit ~ base (B) * thickness (W) * 1/channel radius^2 ($1/R^2$) * concentration fracture strength (C)

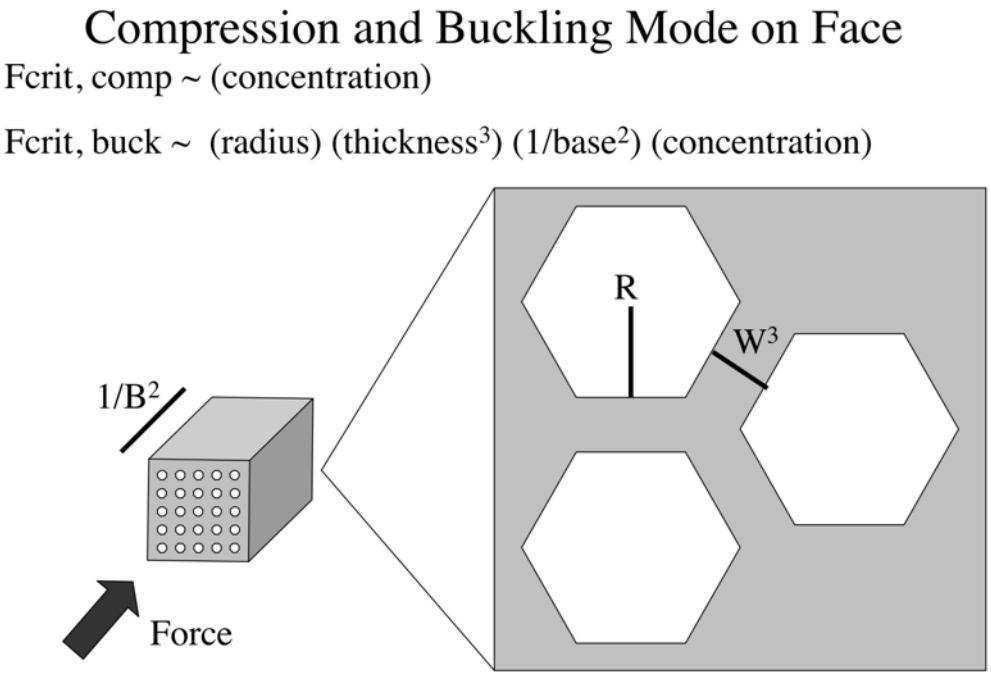

Fig. 4.S4 Buckling mode from the scaffold face. A modification of Euler's buckling is used to model potential methods to strengthen the scaffold design. An increase in channel *base* quickly reduces the scaffold strength. Increasing the wall thickness will most quickly strengthen the scaffold. Near the critical fracture point, the elasticity modulus is approximated as a function of the fracture strength and concentration profile. Thus, Fcrit ~ thickness^3 (W^3) * 1/base^2 ($1/B^2$) * channel radius (R) * concentration fracture strength (C). The honeycomb structure will likely shift the failure mode closer to compression with an increase in concentration.

CHAPTER 5

General discussion

Project overview

Chemoatractants and physical cues can guide cellular growth and extension (Ratner, 1997; Palsson, 2003; Fung, 1993). Cells can release proteins used for navigation of nearby cells. Specifically, diffusible proteins have been shown to guide and support axons of the developing nervous system (Tessier-Lavigne and Placzek, 1991; Tessier-Lavigne, 1994), and further, to guide adult lesioned axons after therapeutic administration into sites of spinal cord injury (Schwab et al., 2002; Grill et al., 1997, Tuszynski and Lu, 2008). Physical interactions with cells have also been shown to affect cellular function, such as migration and orientation. In the central nervous system, *in vivo* microstructures have been shown to affect cellular structure (Tsai et al., 2004; Wong et al., 2008), while *in vitro* axon orientation has been shown to be directed by micron level changes in the mechanical substrate structure (Clark, 1992; Miller et al., 2002; Houchin-Ray et al., 2007).

In addition to a lack of mechanisms for providing physical guidance to injured axons after central nervous system (CNS) injury, lesions in the CNS do not lead to the activation of intrinsic neuronal programs to stimulate a state of regeneration. This is in contrast to molecular events that lead to successful regeneration of axons after lesions in the peripheral nervous system (PNS) (Cajal, 1928). Conditioning lesions (lesions of

the peripheral branch of a dorsal root ganglion (DRG) axon) are one means to enhance the intrinsic growth capacity of DRG neurons after CNS injury (Neumann and Woolfe, 1999; Richardson and Issa, 1984), by enhancing the genetic programs responsible for axon growth and extension (Snider, 2002; Zhou, 2004; Lu, 2004).

We proposed that a combination of chemoattractant cues, physical guidance, and genetic stimulation would promote axon growth across large deficits and into the host tissue beyond the lesion where: 1) The delivery of neurotrophins beyond spinal cord lesion sites using *in vivo* gene delivery could create chemotropic gradients to promote growth of axons into distal host tissue beyond the lesion; 2) The agarose scaffold would promote linearized cellular orientation across the lesion, providing a more uniform substrate for growth factor delivery and axon guidance; and 3) The conditioning lesion could upregulate intrinsic neuronal molecular mechanisms required for axon growth.

This thesis work has demonstrated that combinatorial therapies can promote ascending and descending axon growth across lesion lengths of 2mm. However, upon reaching the distal aspect of the lesion, axons encounter a reactive cell layer, and do not re-penetrate distal spinal cord parenchyma. The scaffold microstructure promoted linear cellular orientation within the channels, while similar non-scaffold treatments had disorganized cellular orientation. Additionally, when crossing lesions of greater length, axon retention was significantly enhanced when the linearized scaffold was used to bridge the lesion instead of a non-organized cell suspension graft. Furthermore, after scaling the existing scaffold technology, a 1cm peripheral nerve

conduit was developed and shown to be biocompatible and to support axon growth *in vivo*.

Discussion

A combinatorial treatment of a chemoattractant and physical guidance with a conditioning lesion promotes long-tract ascending axon guidance in spinal cord injury

The present work began with the observation that *in vivo* gene delivery *beyond* lesion sites expands neurotrophin gradients *beyond* the lesion rather than within it, thus promoting short distance axon regeneration through the lesion boundary and into the host parenchyma (Taylor et al., 2006). Furthermore, a combination of neurotrophic support and a conditioning lesion further promote axon growth beyond the lesion boundary (Blesch et al. 2008). Additionally, a linearized agarose scaffold was previously shown to guide local axons into a large lesion, and incorporation of BDNF secreting cells into the scaffold further enhanced axon growth into the lesion (Stokols et al., 2006). Our aim was to test the hypothesis that the microstructure design of a strictly linear templated agarose scaffold would enhance not just local axon guidance across a lesion site, but would enhance guidance and regeneration of *long-tract* axons into and beyond a spinal cord injury site. Indeed, we found that templated agarose scaffolds can enhance guidance of long-tract *ascending* axons completely through a

spinal cord lesion site, resulting in a 4-fold increase in the number of axons reaching the distal aspect of a lesion cavity compared to a non-organized cell suspension graft. This was a substantial advance over existing cell suspension technologies commonly used in the field of neural repair. Although the regenerating axons crossing the linearized agarose scaffold reach the distal aspect of the lesion, axons do not repenetrate the host parenchyma due to a cellular layer that forms between the scaffold and lesion boundary. Thus, our finding illustrates an advance over existing cellular methods to bridge a lesion site, by providing a means to linearize the tissue microstructure within the normally disorganized spinal cord injury site. However, additional work remains to be done to accomplish re-penetration of these regenerating axons into the host spinal cord.

The nature of the cellular matrix forming between the scaffold implant and host spinal cord was identified by electron microscopic analysis. The cells were identified as primarily fibroblastic, likely migrating from the overlying leptomeninges. These cells may potentially create an inhibitory environment, via secretion of inhibitory molecules, which may prevent axon growth and re-penetration into the host spinal cord. This cell layer did not contain large numbers of inflammatory cells as indicated by Nissl stain, GFAP and IBA1 (granularized tissue, astrocytic marker and microglia, respectively).

A combination of chemoattractant and mechanical guidance cues enhance descending axon growth across a complete transection spinal cord injury

Although our previous work showed that a linearized scaffold enhanced sensory axon regeneration across a lesion, functional recovery is dependent upon regeneration of descending motor axons. Often, different axon tracts respond differently to treatments (such as different growth factors). Work in the first portion of this thesis focused on regeneration of an ascending sensory axonal system in the spinal cord, a model system that was utilized because it is anatomically well-defined, and complete transection of these axons can be reproducibly generated to allow clear assessment of effects of therapeutic manipulations on axonal regeneration. Further, the sensitivity of these axons to the growth factor NT-3 is well-established. Several previous studies in the spinal cord injury field have used this model system to generate mechanistic insight or test candidate therapies. After our initial experiments confirmed that the scaffold supported growth of ascending dorsal column sensory axons, we applied our linearized agarose scaffold to a more complex and challenging paradigm, regeneration of descending axons. Using the growth factor BDNF, some studies have shown *descending* axons to regenerate to the distal aspect of the lesion site (Menei et al., 1998; Bammer et al., 2001). Therefore, we applied a linearized scaffold and a growth factor treatment to test the hypothesis that the combinatorial therapy will enhance axon guidance, thus increasing the number of descending axons extending into and beyond a complete transection.

As previously mentioned, the linearized microstructure design of our agarose scaffolds might guide cellular orientation within the scaffold channels by providing uniform stress and strain distributions for migrating and grafted cells as they form a highly organized cellular channel. Our results indicate that when combined with a growth factor, a templated agarose scaffold markedly enhanced the number of *descending* axons reaching the distal aspect of the lesion compared to the non-organized cell suspension graft. Thus, the scaffold represents a clear advance compared to commonly used cell suspension graft methods. However, axons reaching the distal aspect of the lesion did not repenetrate the host parenchyma. Similar to the scaffolds implanted in the sensory system, this is likely due to a cellular layer forming between the scaffold and host tissue. Although, the combination of a linearized agarose scaffold and a growth factor therapy greatly enhanced descending axon regeneration across a complete transection when compared to suspension grafts in the lesion site, the reactive cell layer and potentially the associated extracellular matrix is clearly the next challenge for further development of the scaffold technology.

Interestingly, after implanting the linearized agarose scaffold, ascending and descending axons were capable of entering the lesion site, but upon reaching the distal aspect of the lesion, they were unable to reenter the host parenchyma. This was likely due to the formation of a predominantly leptomeningeal cell layer between the scaffold and the host parenchyma. Why is it that a cell layer is found on both aspects of the scaffold, but axons are capable of extending into, but not out of the lesion? One possible explanation is that the cell layer is formed after axons have penetrated the

scaffold. A second possible explanation is that the cellular migration occurs early, but does not establish an inhibitory environment, via secretion of extracellular matrix components (ECM), until a later time point, at which the axon reaches the distal aspect of the lesion. A third biomechanical perspective is that due to swelling and cellular migration along the lesion boundary, axon navigation into the lesion may encounter cellular architecture representing a mechanical “funnel”, acting as a stable node for axon penetration, while axons attempting to exit the scaffold may encounter a swollen, abrupt flat surface with only few entry points, representing an unstable node for axon penetration.

The results of this thesis pose an additional question: what mechanisms underlie the synergy between the cellular orientation and growth factor delivery to enhance axon regeneration across a lesion? Clearly, our experiments examining ascending and descending axonal projections resulted in enhanced regeneration across a spinal cord injury with the addition of a linearized agarose scaffold to a growth factor gradient. It appears that growth factor gradients can guide injured axons in a manner similar to guidance mechanisms observed during development. The importance of growth factor gradients for axonal bridging is supported by 2 observations. 1) removal of the growth factor gradient from the linearized scaffold resulted in a significant reduction in axons crossing the lesion site. 2) Even in the absence of a scaffold and despite the cellular disorganization within the lesion, some axonal guidance was retained in the presence of a growth factor gradient and some axons extended for 2mm into the lesion/graft.

Furthermore, the number of ascending axons exiting the scaffold in a spinal cord lesion was enhanced with a conditioning lesion. Specifically, the conditioning lesion enhanced the number of sensory axons exiting a cell seeded scaffold with and without a growth factor gradient, suggesting it promotes axon growth through a separate and synergistic mechanism than the growth factor gradient. It likely that growth associated genes are upregulated within the axons, permitting a greater number of axons to enter the scaffold containing neurotrophins secreted by bone marrow stromal cells. However, there were a greater amount of axons exiting the conditioning lesion scaffold treatment when the growth factor gradient was applied. Thus, it is possible that the conditioning lesion enhances the ability of the ascending axons to detect and respond to NT-3, thus creating a synergistic approach when combined a growth factor gradient.

One additional mechanism underlying the synergistic effects between the growth factor gradient and linearized scaffold might be changes in the diffusion of growth factors and the organization of neurotrophin gradients. A non-uniform ECM deposition is thought to retard molecular diffusion within CNS lesions (Roitbak and Sykova, 1999; Woerly et al., 1999). As our scaffold creates a more uniform cellular organization, the ECM is more uniformly deposited creating a more uniform substrate for growth factor diffusion. Therefore a growth factor gradient can be more uniformly delivered when combined with an organized cellular deposition within linearized scaffold channels, enhancing axonal guidance. Additionally, growth factors may diffuse more easily through the porous agarose walls (refer to Chp.4), reaching the

proximal aspects of the lesion site at concentrations not achieved in the presence of disorganized cell suspension grafts. Therefore, the initial growth factor stimulus for axonal growth cones and the guidance of regenerating axons may be enhanced by a uniform physical substrate for growth factor diffusion.

A scalable, nerve conduit supports axons and vascular penetration after peripheral nerve transection

In the PNS, the current challenges to conduit design are to mimic the regeneration-promoting properties of nerve autografts, particularly at clinically relevant lengths of more than 4mm, and to enhance regeneration and functional recovery after nerve gaps of more than 1cm, a distance, at which recovery with nerve autografts dramatically declines. We have shown our linearized agarose conduit to be biocompatible and support axon growth beyond a clinically relevant length of 4mm. Furthermore, using methods to reduce surface tension and increase agarose diffusion, the design was scaled to longer lesion distances and total available channel volume from 2-10mm and 45-75%, respectively. Depending on the ability of the conduit sleeve to protect the scaffold core from mechanical deformation, the strength of the agarose may be reduced to mimic an autograft, or enhanced to prevent lumen collapse. The strength of the agarose gel was increased via a novel superheating method to increase the agarose gel concentrations from 0.2% to 14%. Additionally, it was confirmed that growth factor diffusion from the scaffold is controlled by the scaffold/host interface. Thus, in theory, growth factors could be delivered in a

controlled manner through external diffusion into the scaffold, such as an external pump connected to the conduit sleeve.

In the conduit design, our aim was to replicate key characteristics of an autologous nerve graft. The linearity within the channels mimics the innate guiding architecture found in the endoneurial tubes and Schwann cells of the nerve autograft. The scaffold channels can be filled with a substrate, which supports or enhances axon growth (stem cells, laminin) to mimic the native cellular substrate of a nerve autograft. Additionally, the scaffold channel and wall sizes can be scaled to adjust the effective lesion volume, potentially enhancing the rate of cellular infiltration of the lesion cavity that is preceding axonal penetration. The fracture strength of the scaffold can be designed to simulate the mechanical properties of the nerve or can be enhanced to provide a mechanically stable conduit. Additionally, growth factors are thought to be released transiently from an implanted nerve autograft. The BSA release study illustrated that the scaffold/host interface would determine the growth factor release profile. As discussed earlier, in theory, a transient growth factors release could be controlled by an external pump connected to the conduit sleeve. Therefore, our current conduit design mimics the "gold standard" autograft key characteristics of: 1) biocompatibility, 2) support for a cellular substrate permissive to axonal extension, 3) vascular penetration, 4) organized cellular orientation, 5) provision of a physical bridge for extracellular matrix formation. 6) durability and flexibility, and 7) potential for transient growth factor delivery.

Future Directions

1) Reduction of reactive cell layer

Although axonal guidance was enhanced across a spinal cord lesion using a linearized agarose scaffold, the axons reaching the distal aspect of the scaffold were unable to penetrate into the host spinal cord parenchyma. This was attributed to the formation of a cellular matrix between the scaffold and host tissue. The cells within this region are likely leptomeingeal fibroblasts, and potentially trap and redirect the linear axon growth away from the host spinal cord. The markers of microglial (IBA1), and granularized tissue (Nissl) indicated only a small immune response to the scaffold implant. In contrast, when a neurotrophin gradient was applied with a cell suspension, without a scaffold, some long-tract ascending axons were capable of growth into the distal host parenchyma. Therefore, the current method of scaffold implantation appears to cause the reactive cell layer to form. As discussed in chapters 2 and 3, future methods to potentially reduce the formation of the reactive cell layer beyond the scaffold are to: 1) Reduce accumulation of fibroblast/leptomeningeal along the edges of the lesion site, by delaying implantation of the scaffold until fibroblast/leptomeningeal activation is reduced (Ross et al., 1968; Conrad et al., 2005; Popovich et al., 1997). 2) Place a matrix barrier, such as an agarose thin film, along the top of the scaffold and dorsal host tissue, to impede the migration of leptomeningeal cells into the lesion site. 3) Apply anti-mitotic agents to reduce the division and migration of leptomeningeal cells or fibroblasts into the site of scaffold

implantation (Pruss et al., 1979; Brockes et al., 1979). 4) Implant a scaffold with an elasticity modulus more closely mimicking the spinal cord, such as the 0.6% agarose concentrations. During movement of the spine, this may reduce potential mechanical agitation of the host tissue interface, which may enhance leptomeningeal migration or ECM deposition. 5) The scaffold tips may be coated with poly(ethylene glycol) (PEG) to promote cellular solubility along the host interface, reducing cellular migration between the host wall and the scaffold interface (Shi et al., 1999; Norwood et al., 1976; Lentz, 1994).

2) Biodegradable implant

One important aspect of biomaterial design is the capability for the implant to be absorbable into the body (Ratner, 1997). Although our agarose scaffold is biocompatible and supports robust axon growth, it is not absorbable. In spinal cord and peripheral nerve applications, a biodegradable scaffold would eventually provide a more mechanically homogenous interface upon reabsorbtion and replacement by glia, while additionally increasing the eventual cross sectional area for axon penetration. Mold and etching processes can be adapted to produce a variety of casts. Thus theoretically, our templated agarose process is capable of casting many materials (we have casted a preliminary alginate scaffold). We have already shown our agarose hydrogel to be biocompatible, a characteristic that may be attributed to the manner in which water molecules present themselves at the scaffold interface (Ratner, 1997). Thus, it would be ideal to create a similar hydrogel with biodegreadable

characteristics. Future experiments could explore use of an alginate hydrogel, which slowly degrades as the calcium ions are released from the structure. Using the templated scaffold technology, preliminary alginate scaffolds have been casted. Alternatively, our scaffold design could be adapted to cast synthetic biodegradable polymers such as Matrigel, fibronectin mats, fibrin glue, poly(alpha-hydroxy acids), and polyethylene glycol, which have all previously been shown to support axon growth in the spinal cord (Novikova et al., 2003). However, the current chemicals in the etching steps of the manufacturing methods will have to be adapted to match the biomaterial of choice. Thus, by adapting our current technology to use alginate or other biodegradable hydrogels, biodegradable scaffolds should be developed and tested *in vivo*. We are currently adapting the process to a biodegradable alginate gel by optimizing the chemicals involved in the etching step to prevent calcium release and scaffold degradation, during manufacturing.

3) Scale-up of the peripheral nerve conduit

The peripheral nerve conduit offers a large array of future directions. Clinically, there is a sharp drop in functional recovery if nerve gaps extend beyond 1cm, thus there is a need to scale the scaffold beyond a length of 1cm. The scaffold lumen could be filled with a variety of growth promoting substrates, such as L1, N-cadherin and laminin (Lemmon et al., 1992). In addition the effective lesion volume can be reduced by scaling the channel and wall sizes within the conduit, reducing the time associated with cell infiltration within the cavity, potentially increasing the rate of

initial axon growth. Additionally, an alternate conduit sleeve could be tested with more elastic properties (i.e. collagen sheet) to more closely mimic the elasticity of a nerve autograft. Furthermore, axon regeneration might be enhanced by transient delivery of growth factors to the scaffold core via an osmotic pump (i.e. Alzet). Taken together, our experiments have provided the basis for the development of a peripheral nerve conduit that may exceed the properties of nerve autografts.

Conclusions

Treatment strategies that promote axon linearity and retention are essential to achieving functional recovery after large lesion SCI. The present work demonstrates that delivery of neurotrophic factors, cell body gene upregulation, and microstructure cellular guidance, may be used as part of an overall growth-promoting and guidance strategy to support regeneration of injured axons. Although axons did not regenerate beyond the host lesion tissue, there were a greatly enhanced number of axons reaching the distal end of the large lesions. For these reasons, a combination of chemical and physical guidance cues may be important to enhance regeneration after injuries in the peripheral nervous system and in the injured spinal cord.

Acknowledgement

This work was made possible by grants from the NIH. I would like to thank

Dr. Jeff Sakamoto, Dr. Leif Havton, Dr. Armin Blesch, and Dr. Mark Tuszynski for work on this project.

References

Bamber, N, H Li, X Lu, M Oudega, P Aebischer and X Xu (2001) Neurotrophins bdnf and nt-3 promote axonal re-entry into the distal host spinal cord through schwann cell-seeded mini-channels. European Journal of Neuroscience 13(2): 257-68.

Blesch, A, H Uy, N Diergardt and M Tuszynski (2000) Neurite outgrowth can be modulated in vitro using a tetracycline-repressible gene therapy vector expressing human nerve growth factor. Journal of Neuroscience Research 59: 402-09.

Blesch, A, P Lu and M Tuszynski (2002) Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. Brain Research Bulletin 57(6): 833-38.

Blesch, A (2008) Condtioning lesion and neurophic delivery act synergistically in promoting ascending axons into site of spinal cord injury. SFN Abstract.

Brockes, J (1981) Studies on cultured schwann cells: The induction of myelin synthesis, and the control of their proliferation by a new growth factor. Journal of Experimental Biology 95(1): 215-30.

Cai, D, Y Shen, M De Bellard, S Tang and M Filbin (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by mag and myelin via a camp-dependent mechanism. Neuron 22: 89-101.

Cajal, R S.(1928) degeneration and regeneration of the nervous system. Hafner, New York.

Clark, P (1992) Cell guidance by micropatterned adhesiveness in vitro. Journal of Cell Science 103(1): 287-92.

Costigan, M and C Woolf (2002) No dream, no pain closing the spinal gate. Cell 108(3): 297-300.

Costigan, M, K Befort, L Karchewski, R Griffin, D D'urso, A Allchorne, J Sitarski, J Mannion, R Pratt and C Woolf (2004) Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. feedback.

Forman, D, I Mcquarrie, F Labore, D Wood, L Stone, C Braddock and D Fuchs (1980) Time course of the conditioning lesion effect on axonal regeneration. Brain Res 182(1): 180-5.

Forman, D, I Mcquarrie, F Labore, D Wood, L Stone, C Braddock and D Fuchs (1980) Time course of the conditioning lesion effect on axonal regeneration. Brain Res 182(1): 180-5.

Fung, Y (1993). Biomechanics: Mechanical properties of living tissues, Springer.

Grill, R, K Murai, A Blesch, F Gage and M Tuszynski (1997) Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. Journal of Neuroscience 17(14): 5560-72.

Hadlock, T, C Sundback, D Hunter, M Cheney and J Vacanti (2000) A polymer foam conduit seeded with schwann cells promotes guided peripheral nerve regeneration. Tissue Engineering 6(2): 119-27.

Hoffman, A (2002) Hydrogels for biomedical applications. Advanced Drug Delivery Reviews 54(1): 3-12.

Houchin-Ray, T, L Swift, J Jang and L Shea (2007) Patterned plg substrates for localized DNA delivery and directed neurite extension. Biomaterials 28(16): 2603-11.

Lee, K and D Mooney (2001) Hydrogels for tissue engineering. Chemical Reviews 101(7): 1869-80.

Lemmon, V, S Burden, H Payne, G Elmslie and M Hlavin (1992) Neurite growth on different substrates: Permissive versus instructive influences and the role of adhesive strength. Journal of Neuroscience 12(3): 818-26.

Li, S, S Archibald, C Krarup and R Madison (1992) Peripheral nerve repair with collagen conduits. Clinical materials 9(3-4): 195-200.

Lindsay, R (1988) Nerve growth factors (ngf, bdnf) enhance axonal regeneration but are not required for survival of adult sensory neurons. Journal of Neuroscience 8(7): 2394-405.

Lu, B (2004) Acute and long-term synaptic modulation by neurotrophins. NGF and Related Molecules in Health and Disease.

Lu, P, H Yang, L Jones, M Filbin and M Tuszynski (2004) Combinatorial therapy with neurotrophins and camp promotes axonal regeneration beyond sites of spinal cord injury. Journal of Neuroscience 24(28): 6402-09.

Lu, P and M Tuszynski (2008) Growth factors and combinatorial therapies for cns regeneration. Experimental Neurology 209(2): 313-20.

Manfredi, J and S Horwitz (1984) Taxol: An antimitotic agent with a new mechanism of action. Pharmacol Ther 25(1): 83-125.

Matsumoto, K, K Ohnishi, T Kiyotani, T Sekine, H Ueda, T Nakamura, K Endo and Y Shimizu (2000) Peripheral nerve regeneration across an 80-mm gap bridged by a polyglycolic acid (pga)–collagen tube filled with laminin-coated collagen fibers: A histological and electrophysiological evaluation of regenerated nerves. Brain Research 868(2): 315-28.

Mcquarrie, I (1978) The effect of a conditioning lesion on the regeneration of motor axons. Brain Res 152(3): 597-602.

Menei, P, C Montero-Menei, S Whittemore, R Bunge and M Bunge (1998) Schwann cells genetically modified to secrete human bdnf promote enhanced axonal regrowth across transected adult rat spinal cord. European Journal of Neuroscience 10(2): 607-21.

Miller, C, S Jeftinija and S Mallapragada (2002) Synergistic effects of physical and chemical guidance cues on neurite alignment and outgrowth on biodegradable polymer substrates. Tissue Engineering 8(3): 367-78.

Neumann, S and C Woolf (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23(1): 83-91.

Nikulina, E, J Tidwell, H Dai, B Bregman and M Filbin (2004) The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. Proceedings of the National Academy of Sciences 101(23): 8786-90.

Novikova, L, L Novikov and J Kellerth (2003) Biopolymers and biodegradable smart implants for tissue regeneration after spinal cord injury. Current Opinion in Neurology 16(6): 711.

Oudega, M, S Varon and T Hagg (1994) Regeneration of adult rat sensory axons into intraspinal nerve grafts: Promoting effects of conditioning lesion and graft predegeneration. Exp Neurol 129(2): 194-206.

Oudega, M and T Hagg (1996) Nerve growth factor promotes regeneration of sensory axons into adult rat spinal cord. Experimental Neurology 140(2): 218-29.

Palsson, B (2003). Tissue engineering, CRC Press.

Patapoutian, A and L Reichardt (2001) Trk receptors: Mediators of neurotrophin action. Current Opinion in Neurobiology 11(3): 272-80.

Peppas, N, P Bures, W Leobandung and H Ichikawa (2000) Hydrogels in pharmaceutical formulations. European Journal of Pharmaceutics and Biopharmaceutics 50(1): 27-46.

Qiu, J, D Cai, H Dai, M Mcatee, P Hoffman, B Bregman and M Filbin (2002) Spinal axon regeneration induced by elevation of cyclic amp. Neuron 34(6): 895-903.

Qiu, J, W Cafferty, S Mcmahon and S Thompson (2005) Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. Journal of Neuroscience 25(7): 1645-53.

Ratner, B (1997). Biomaterials science: An introduction to materials in medicine, Academic Press.

Reichardt, L (2006) Neurotrophin-regulated signalling pathways. Philosophical Transactions of the Royal Society B: Biological Sciences 361(1473): 1545-64.

Richardson, P and V Issa (1984) Peripheral injury enhances central regeneration of primary sensory neurones. Nature 309(5971): 791-93.

Roitbak, T and E Sykova (1999) Diffusion barriers evoked in the rat cortex by reactive astrogliosis. Glia 28: 40-48.

Ronsyn, M, Z Berneman, V Van Tendeloo, P Jorens and P Ponsaerts (2008) Can cell therapy heal a spinal cord injury? Spinal Cord.

Ross, R (1968) The fibroblast and wound repair. Biological Reviews 43(1): 51-91.

Sabine Conrad, T, H Schluesener, M Adibzahdeh and J Schwab (2005) Spinal cord injury induction of lesional expression of profibrotic and angiogenic connective tissue growth factor confined to reactive astrocytes, invading fibroblasts and endothelial cells. J. Neurosurg Spine 2: 319-26.

Sandvig, A, M Berry, L Barrett, A Butt and A Logan (2004) Myelin-, reactive glia-, and scar-derived cns axon growth inhibitors: Expression, receptor signaling, and correlation with axon regeneration. Glia 46(3): 225-51.

Schwab, M (2002) Repairing the injured spinal cord. Science 295(5557): 1029-31.

Seijffers, R, A Allchorne and C Woolf (2006) The transcription factor atf-3 promotes neurite outgrowth. Molecular and Cellular Neuroscience 32(1-2): 143-54.

Seijffers, R, C Mills and C Woolf (2007) Atf3 increases the intrinsic growth state of drg neurons to enhance peripheral nerve regeneration. Journal of Neuroscience 27(30): 7911.

Snider, W, F Zhou, J Zhong and A Markus (2002) Signaling the pathway to regeneration. Neuron 35(1): 13-16.

Spencer, T and M Filbin (2004) A role for camp in regeneration of the adult mammalian cns. Journal of Anatomy 204(1): 49-55.

Sroga, J, T Jones, K Kigerl, V Mcgaughy and P Popovich (2003) Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. The Journal of Comparative Neurology 462(2): 223-40.

Stokols, S, J Sakamoto, C Breckon, T Holt, J Weiss and M Tuszynski (2006) Templated agarose scaffolds support linear axonal regeneration. Tissue Engineering 12(10): 2777-87.

Stokols, S and M Tuszynski (2006) Freeze-dried agarose scaffolds with uniaxial channels stimulate and guide linear axonal growth following spinal cord injury. Biomaterials 27(3): 443-51.

Taylor, L, L Jones, M Tuszynski and A Blesch (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. Journal of Neuroscience 26(38): 9713.

Tessier-Lavigne, M and M Placzek (1991) Target attraction: Are developing axons guided by chemotropism? Trends Neurosci 14(7): 303-10.

Tessier-Lavigne, M (1994) Axon guidance by diffusible repellants and attractants. Curr Opin Genet Dev 4(4): 596-601.

Tsai, E, P Dalton, M Shoichet and C Tator (2004) Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection. Journal of Neurotrauma 21(6): 789-804.

Verma, P, S Chierzi, A Codd, D Campbell, R Meyer, C Holt and J Fawcett (2005) Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. Journal of Neuroscience 25(2): 331-42.

Woerly, S, P Petrov, E Sykova, T Roitbak, Z Simonova and A Harvey (1999) Neural tissue formation within porous hydrogels implanted in brain and spinal cord lesions: Ultrastructural, immunohistochemical, and diffusion studies. Tissue Eng 5(5): 467-88.

Wong, D, P Krebsbach and S Hollister (2008) Brain cortex regeneration affected by scaffold architectures. Journal of Neurosurgery 109(4): 715-22.

Yiu, G and Z He (2006) Glial inhibition of cns axon regeneration. Nature reviews. Neuroscience(Print) 7(8): 617-27.

Zhou, F, M Walzer and W Snider (2004) Turning on the machine genetic control of axon regeneration by c-jun. Neuron 43(1): 1-2.