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Angiogenesis in Response to Varying Fiber Size in an Electrospun Scaffold In Vivo.

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Angiogenesis in Response to Varying Fiber Size in an Electrospun Scaffold In Vivo

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

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List of Abbreviations

3D	Three Dimensional
CNS	Central Nervous System
FFT	Fast Fouier Transform
HIF	Hypoxia Inducing Factor
HFIP	Hexafluoroisopropanol
LM	Light Microscopy
NGS	Normal Goat Serum
PBS	Phosphate-Buffered Saline
PDS	Polydioxanone Suture
PGLA	Polyglycolic-Lactic Acid
RECA-1	Rat Endothelial Cell Antigen – 1
ROS	Reactive Oxygen Species
SCI	Spinal Cord Injury
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

ANGIOGENESIS IN RESPONSE TO VARYING FIBER SIZE IN AN ELECTROSPUN SCAFFOLD IN VIVO

Damien Ellis Brown, Master of Science

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

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Injury to the spinal cord results in partial or complete loss of sensory perception and motor function. After spinal cord injury (SCI), damaged tissue dies and a cavity will form. This cavity prevents the regeneration of tissue and any functional recovery. One way to address the cavity is the insertion of an electrospun scaffold that our lab has created. This provides a substrate for regenerating tissue to grow on, and it is thought that reestablishing the blood supply within the scaffold will allow cells necessary for

regeneration to thrive. This could ultimately lead to meaningful recovery for patients who have suffered SCI. Full spinal cord transections were performed on rats, and the scaffolds were inserted into the lesion site. Two different types of scaffold were tested to see if altering the fiber size in the scaffolds produced more blood vessels, and ultimately better regeneration of tissue.

Chapter 1

Spinal Cord Injury, Related Pathology, and the Role of Angiogenesis in Recovery

Synopsis:

Defining Spinal Cord Injury

Spinal cord injury (SCI) occurs when there is an insult to the structures composing the spinal cord, causing disruption to the signaling pathways between the brain and the rest of the body. Depending on the level and severity of the injury, there is typically a loss of sensory information and motor function below the site of the lesion. This is a direct result of the severing of axons, the neuronal structures which carry information to and from the brain, and the death of cells which surround axons and supports their proper function. Specifically, disruption to the vasculature results in loss of trophic and metabolic support needed for proper cellular function.

Immediately following the initial insult to the spinal cord tissue, there are a host of secondary events which cause further damage resulting in an inability of the cells to regenerate. As is typical in most injuries, there is an innate immune and inflammatory response in the injured area. With regard to the central nervous system (CNS), injury to the spinal cord results in a far greater immune response than similar injuries to the

brain. It has been shown that SCI results in 2-4 times the amount of macrophage, lymphocyte, and neutrophil infiltration than similar injuries to the brain, illustrating the very delicate and sensitive nature of this structure (Schnell, Fearn, Klassen, Schwab, & Perry, 1999).This immune response releases many substances which drive the neurons and supporting cells (glia) into programmed cell death (Emery et al., 1998). The death of these cells leaves behind cellular debris which elicits further immune response, as well as preventing axonal regeneration. In addition to this, glial cells known as astrocytes surround the injury site activate, become enlarged and divide rapidly creating a scar which encases the damaged tissue (Figure 1.1). This scar acts as a barrier that not only physically inhibits axon growth, but the cells composing the scar release compounds that further block regeneration (Sofroniew & Vinters, 2010). As the damaged cells within the scar die off and are cleared away, the result is an acellular environment which fills with fluid presenting a physical gap which further retards regeneration (Fawcett, 2006).

The complex nature of these events complicates the treatment of SCI tremendously. Many approaches are being explored which attempt to address and overcome these obstacles. Utilizing different combinatorial strategies helps to mitigate the initial inflammatory response and facilitate functional recovery. This thesis will explore the use of an electrospun scaffold as a therapeutic strategy to overcome some of the obstacles of SCI. Using a complete spinal cord transection model in rats replicates many of the issues that are present in different types of SCI. We will examine the histology associated with the implant, focusing on the angiogenic response and the ability of the scaffold to direct the establishment of a new vascular supply. We will first

examine the anatomy of the spinal cord and importance of the blood supply. We will then introduce types of SCI and related pathology. Then we will look closer at the factors which prevent regeneration in the injured spinal cord, and the ways in which our therapeutic strategy addresses them. We will later compare the normal blood supply in spinal cord tissue to the re-establishment of new vasculature through the implanted electrospun scaffold.

Introduction:

Anatomy of the Spinal Cord

The human spinal cord is one of the most complex cellular networks in the body, composed of over one billion neurons. The human spinal cord is an ovoid grouping of neurons, and is approximately 45cm in length. It begins just below the medulla oblongata near the foramen magnum of the skull, and extends all the way down through the spinal column terminating in the upper lumbar region. There are two distinct regions that compose the majority of the spinal cord; the white and gray matter. White matter refers to the outermost area of the spinal cord which is composed of efferent and afferent axonal tracts. These tracts are responsible for relaying the motor signals leaving the brain, and the sensory information returning from the periphery. Damage to the white matter is commonly seen in SCI, and is the primary reason for the loss of sensation and function. The gray matter is located in the core of the spinal cord and houses the neuronal cell bodies of the axons. These cell bodies play a central role in motor control, central pattern generation, and reflex loops.

The spinal cord maintains a uniform thickness except in two regions, the cervical and lumbar enlargements. These two regions have a greater amount white matter due to the fact that these are the levels at which innervation of both the upper and lower limbs take place. Due to its sensitive nature, there are several protective elements which surround the spinal cord to protect it from injury. The three meninges of the brain, the dura mater, aracnoid mater, and the pia mater also extend down into the spinal canal encapsulating the spinal cord. Cerebrospinal fluid surrounds the spinal cord acting as a shock absorber, preventing the spinal cord from hitting the walls of the surrounding spinal column.

Spinal Cord Blood Supply

The vascular network which feeds the spinal cord is an integral piece of the neural network. It supplies oxygen and other necessary nutrients to the cells within the spinal cord, as well as carrying away metabolic waste. The blood supply to the spinal cord originates from the vertebral arteries, thoracic and lumbar segmental arteries, and deep and ascending cervical arteries. The anterior spinal artery branches off of the vertebral arteries, running the entire length of the spinal cord (Martirosyan et al., 2011). The anterior spinal artery supplies the anterior two thirds of the spinal cord along with central core of gray matter. The posterior spinal arteries branch into the pial circumferential arteries which supply the remaining one third, the posterior spinal cord and peripheral white matter. The anterior and posterior spinal arteries anastomose around the conus medularis in what is known as the arterial basket (Lamin and Bhattacharya 2003). (Figure 1.2)

The Blood Spinal Cord Barrier

The cardiovascular system in humans is a complex network which distributes blood throughout the body to the various tissues and organ systems. The vascular supply is responsible for many homeostatic processes such as gas exchange, nutrient delivery and pH balance. When one examines the vascular supply associated with the Central Nervous System (CNS) as opposed to rest of the body, there is one notable difference. Due to the sensitive nature of the brain, spinal cord, and associated nervous tissue, the body has evolved a specialized vascular network with strict regulation of exchange between the blood and the interstitial fluid of the CNS. This particular feature of the CNS vascular supply is known as the Blood Brain/ Blood Spinal Cord Barrier (BBB/BSCB). For the purposes of this paper, we will be referring to the BSCB. The BSCB is the network which regulates what passes from the blood into the spinal cord, as well as prohibiting immune cells access to the brain and spinal cord. Often with contusional SCI, there is disruption to the BSCS which allows infiltration of immune cells and exposure to neurotoxic compounds. Along with the reestablishment of the vasculature, repair of the BSCB is also a crucial step in creating an environment that is conducive to regeneration and proper neurologic function.

The arteries of the spinal cord turn into arterioles as they enter the parenchyma of the spinal cord, and end when they reach the level of the capillaries. The BSCB is present at the capillary level and is maintained by specialized endothelial cells (ECs) that share tight junctions with neighboring ECs. Other cells which interact with these EC's also contribute to the BSCB and include astrocytes, pericytes, and neuronal processes. Together they form what is known as the neurovascular unit (Figure 1.3),

and all play a role in the regulation of blood flow and membrane transport of substances into and out of the spinal cord microenvironment (Benarroch, 2012).

Classification of Spinal Cord Injury

The term spinal cord injury is a broad classification of the very complex, widely varied types of injuries which exist. There are a number of different ways that an individual can sustain damage to the spinal cord; insults to the undamaged cord (Figure 1.4A) include squeezing, crushing or tearing of the cord, or more invasive means such as lacerations from objects penetrating the spinal column. For these reasons, more specific classes were created in order to identify the gross appearance, means in which the injury was sustained, and the associated histology. There are four distinct categories into which an injury can be classified; solid cord injuries, contusion/cavitation injuries, laceration injuries, and massive compression injuries.

Solid Cord Injury

This class of SCI accounts for approximately 10% of all cases. From a gross anatomical perspective the spinal cord looks normal, but when examined at a histological level a loss of typical cellular architecture is seen (Figure 1.4B). With most SCI the cause of the injury is readily apparent; with solid cord injuries the exact cause is not fully understood. It is believed that this type of SCI has an underlying inflammatory component which results in the loss of the delicate cellular networks within the spinal cord. The primary insult which causes secondary inflammation is thought to be from some form of mechanical stress, such as sudden stretching or squeezing of the spinal cord (Norenberg, Smith, & Marcillo, 2004).

Contusion/Cavitation Injury

This form of SCI is the most common, representing approximately 50% of all cases encountered. The cause of contusion/ cavitation injuries occur when the vertebra of the spinal column or the vertebral discs are dislocated and press against the spinal cord causing bruising. Being that there is no physical penetration in this type of injury, the protective meningeal layers are left intact. Despite the lack of tissue disruption during the initial insult, bleeding within the central gray matter leads to an inflammatory response and ultimately necrosis of the tissue. Once dead tissue has been removed by the inflammatory cells, the result is a fluid filled cyst (Figure 1.4C). Along with this, reactive astrocytes begin to proliferate and surround the damaged area, resulting in the formation of what is known as the gliotic scar (Norenberg et al., 2004).

Laceration Injury

The laceration injury represents nearly 20% of SCI cases seen. This type of injury can be caused when broken pieces of a damaged vertebra pierce the meninges and damage the spinal cord (Figure 1.4D). Other causes of this type of injury are from foreign objects penetrating the spinal column; often this is the result of violent acts such as gun shots and stabbings. The disruption of the meninges allows for infiltration of cells not normally present in the spinal cord, particularly influx of fibroblasts which begin to deposit collagen. This deposition leads to the formation the mesenchymal scar, which hinders the ability of axons to regenerate (Norenberg et al., 2004).

Massive Compression Injury

This type of SCI accounts for a little less than 20% of SCI cases reported. As the name suggests, massive compression injuries occur when the spinal cord is crushed by some physical force, usually a fall or blunt force trauma. As is seen with laceration injuries (Figure 1.4E)., the meninges are often damaged leading to similar fibroblastic invasion and scar formation (Norenberg et al., 2004).

Clinical Classification

The functional outcome of a SCI is what determines the clinical classification. The classification categories are known as complete, incomplete or discomplete. A complete injury is one in which total loss of motor function and sensory information is experienced below the site of the lesion. In an incomplete injury, some form of sensory or motor function is retained indicating that some of the axonal tracts have been left intact. The final classification is what is known as discomplete, and is the most misunderstood of the three. This is when an injury appears to be complete, yet there is still some conscious control over areas below the site of the lesion.

SCI Pathology

The most common form of SCI experienced is the contusion/cavitation injury. It is for this reason that our has uses a complete spinal cord transection which reflects the type of problems encountered with this type of injury. There are many physical limitations associated with SCI that must be overcome, and we will examine these limitations in upcoming sections.

Immediately after a traumatic injury, the spinal cord experiences inflammation and edema. This inflammatory cascade leads to cell death in the area of inflammation.

The severing of neuronal cells from there source of neurotrophic support also contributes to the massive cell death associated with SCI. A process known as reactive astrogliosis results in the formation of an impenetrable network of cells which ultimately result in scar formation. Also known as the gliotic scar, this tight grouping of astrocytes presents a physical barrier which regenerating axons cannot pass (Sofroniew, 2005). Cells within the scar undergo a process known as liquefactive necrosis, ultimately leading to the formation of the fluid filled cyst. The cyst itself is the ultimate barrier to regeneration being that it is a physical gap in the tissue, providing no structure on which axons and blood supply can regenerate.

Reactive Astrogliosis and the Gliotic Scar

Astrocytes are glial cells found throughout the nervous system which play many complex supportive roles. They participate in such processes as synaptic function, blood flow regulationand ionic balance. Astrocytes also play a key role in the proper functioning of the BBB/BSCB. When the CNS is subjected to an insult, astrocytes surrounding the injury site change morphology and begin a process which is referred to as reactive astrogliosis. During this process, the cells become enlarged and begin to divide rapidly. The reactive cells begin to amass around the injury site forming a dense cellular structure called the gliotic scar (Sofroniew, 2009). The reactive astrocytes play a key role in restructuring the spinal cord architecture by creating new extracellular matrix (ECM). One of the most important functions of the new ECM is to provide a framework for endothelial cells to begin forming new vasculature (White & Jakeman, 2008). It is this vascular supply which provides the metabolic support necessary for survival of neural tissue, and is necessary for any meaningful recovery. The gliotic scar is a

protective mechanism which helps to minimize damage and further loss of tissue, but it also has many negative affects which prevent the regeneration of axons.

The processes of the reactive astrocytes form tight junctions with one another producing what is known as the glial limitans. This resulting barrier is instrumental in separating normal from damaged tissue, as well as preventing the spread further necrosis. This protective barrier, however, also acts as a wall through which regenerating axons and blood vessels cannot cross. One particular compound that is released by cells of the gliotic scar is known as chondroitin sulfate proteoglycan (CSPG). Proteoglycans are structural elements consisting of a protein core with polysaccharides attached along its length. CSPGs are glycosaminoglycans which have been sulfated, and have been shown to inhibit the regeneration of axons (White & Jakeman, 2008).

The Fluid-filled Cyst

One of the major barriers to overcome after a contusional SCI is the formation of the fluid-filled cyst. The cyst formation takes place over the course of a few weeks to months after the initial injury. The size and number of cysts present can vary depending on the extent of the damage sustained. The cysts form in the damaged area contained within the gliotic scar, and are the result of the removal of the cellular debris. This fluid filled acellular cavity has no inherent structure on which regenerating cells can grow, and prevents revascularization and axonal regeneration from taking place (Cholas, Hsu, & Spector, 2012)(Hagg & Oudega, 2006).

Vascular Disruption following SCI

Damage to the blood supply in the spinal cord is thought to be the key factor in the progression of degenerative effects seen in SCI. Hemorrhaging of damaged vessels disrupts nutrient and oxygen delivery to cells, leading to cell death (Rooney et al., 2009). Along with this initial insult, secondary injury can occur to surrounding tissue that would otherwise remain intact. The compromised BSCB allows inflammatory molecules and immune cells access to neurons causing further cell death (Whetstone, Hsu, Eisenberg, Werb, & Noble-Haeusslein, 2003). In addition to this, the response of the cardiovascular system to SCI includes decreased cardiac output and bradycardia, along with hypotension resulting in further ischemia (Mautes, Weinzierl, Donovan, & Noble, 2000). One of the major impediments to axonal regeneration and functional recovery is this damage to the vascular network. Without the recovery of this crucial element, cell survival and proliferation cannot occur.

Treating SCI: Inducing angiogenesis as a therapeutic strategy

Within the realm of SCI research, the focus of most therapeutic strategies is the regeneration of axons. While this is a necessary step to meaningful recovery, the importance of reestablishing a properly functioning blood supply is very often neglected. Understanding the angiogenic process and applying this information in a therapeutic strategy may be one of the greatest and most overlooked tools available in treating SCI and may other disease states.

The Mighty Endothelial Cell

Throughout the vascular system, at the core of any vessel there is the endothelial cell. ECs line the inside of all vessels throughout mammalian circulatory system, and it

was once believed that ECs were a cell type of little importance with little variation. Upon further examination ECs have been shown to vary greatly in size, orientation, appearance, and function. ECs participate in many essential physiological functions including vasoconstriction and dilation, fibrinolysis, antigen presentation, catabolism of lipoproteins, blood coagulation, transportation of blood cells, adaptive and innate immunity, and angiogenesis (Aird, 2012).

The phenotype of ECs can vary widely between organs, vessel types, and even neighboring ECs. They can be very tightly arranged in a fashion referred to as continuous, or loosely arranged in what is known as fenestrated as well as discontinuous. These types of orientation are directly related to the needs of the particular system and its associated tissues. In large arterial sections ECs are arranged along the longitudinal axis of blood flow in order to cope with the shear stress associated with the large volumes and pressures, as opposed to those found in veins (Flaherty et al., 1972). In the CNS, ECs have stronger tight junctions and highly regulated channels to accommodate its strict metabolic requirements. In systems such as the kidneys, ECs are assembled in a fenestrated fashion allowing for the filtration of the blood.

Understanding the role ECs play in regenerative as well as well as disease states is something that is essential in treating SCI as well as many other maladies present in a clinical setting. When treating many diseases, the focus tends to be more macroscopic in scale; more attention is paid to the vessels or the blood due to their accessibility. Due to the tight association of ECs with each other and the lumen of the vessel, ECs are difficult to isolate and study. Due to these complications, the great

importance and possible wealth of therapeutic techniques involving the endothelium has been largely ignored.

Angiogenesis vs. Vasculogenesis

There is often much confusion and debate about the difference between angiogenesis and vasculogenesis. It is important to have an understanding of the two in order to have an appreciation for the roles that each one plays in a biological system. While there is some interplay between them early on in development, for the most part angiogenesis and vasculogenesis are two distinct processes.

Vasculogenesis is described as the series of events that take place during the establishment of the initial capillary plexus. During vasculogenesis, mesodermally derived cells differentiate into angioblasts or hemangioblasts, and then further differentiate into endothelial cells and hematopoietic cells respectively. There is then an organization of ECs into the primary capillary plexus (Pepper, Mandriota, Vassalli, Orci, & Montesano, 1996).

Angiogenesis is a process that takes place after the initial establishment of a vascular network. This process involves the formation of newly formed vessels via sprouting or seeding from pre-established blood vessels. Angiogenesis takes place during many different developmental processes, such as the formation of blood vessels in the placenta, or in other physiologic states such as the formation of the corpus lutem during ovulation in females. Angiogenesis can also be associated with many pathological states, such as in tumor formation or rheumatoid arthritis (Pepper et al., 1996). One of the most important functions of angiogenesis is its involvement in

maintenance and preservation of an organism. In processes such as inflammation, wound healing or response to an ischemic event, angiogenesis is of the utmost importance (Cassell, Hofer, Morrison, & Knight, 2002).

Angiogenesis in response to injury

There are a host of factors which are a part of the angiogenic response post injury. Two major angiogenic processes can occur following an injury; one involves vessel sprouting from existing vessels, while the other arises through the seeding of circulating EC's, and is referred to as non-sprouting (Risau, 1997). During the EC seeding process, one major factor involved is the Vascular Endothelial Growth Factor (VEGF). Following spinal cord injury, vascular disruption causes the cells in the spinal cord to become hypoxic releasing hypoxia-inducible factor (HIF). The mechanism that is believed to trigger the release of HIF is the production of reactive oxygen species (ROS) from the mitochondria within the hypoxic cells. These compounds are required for HIF transcription factors to increase gene expression for VEGF release (Chandel et al., 2000). The VEGF then binds to VEGF receptors on endothelial cells, causing a tyrosine kinase cascade. This cascade triggers the breakdown of the basal lamina of existing blood vessels, allowing the release of ECs and the seeding of new vessels within the surrounding tissue (Risau, 1997).

During the sprouting angiogenic process, the extracellular matrix is degraded and proliferation and migration of endothelial cells occur. The sprouting occurs at the ends of the existing vessels, and these sprouts produce long extensions of the endothelial cells toward the areas requiring revascularization (Mackenzie & Ruhrberg, 2012). It is

believed that cells producing VEGF produce the chemotactic gradient which directs the sprouting. However, in either case revascularization is hindered by the physical gap produced by the cystic cavity, and requires some type of substrate on which both the seeding and sprouting processes can occur.

Engineering angiogenesis

The reestablishment of a functional blood supply is a necessary step on the road to regeneration. Angiogenesis is a mechanism that the body uses in response to injuries, and is crucial in wound healing. However, care must be taken in the amount of angiogenesis that occurs as well as the quality of the vessels that are created.

With regard to the spinal cord, the newly formed vasculature should parallel that of the original blood supply. The ideal result would be similar vessel distribution, as well as comparable vessel permeability. The permeability of the newly formed vessels is important to consider due to the fact that the vessels in the spinal cord have very low permeability due to the BSCB. Some disease states such as multiple sclerosis are associated with increased vessel permeability (D'Aversa, Eugenin, Lopez, & Berman, 2012). The ideal therapy would address the physical limitations associated with SCI, along with promoting regeneration that is similar in architecture to the original tissue.

The Neuron-vascular Link

During embryonic development, the nervous system and the vascular plexus share a very close association. The formation of the nervous system undergoes many stages of remodeling before reaching maturity, as is the case with the vasculature. Both systems experience a rapid formation, followed by a phase of reshaping and pruning to

achieve the proper distribution (Arese, Serini, & Bussolino, 2011). As the CNS is developing, the absence of vascular precursor cells makes the infiltration of blood vessels a critical process for continued development and cell survival. Crosstalk between the nervous and vascular systems is a process that helps to establish the intricate association of the two systems throughout life (Bautch & James, 2009).

Previous studies have shown that cells purified from the bone marrow have the ability to differentiate into neurons in the human brain (Mezey et al., 2003). It has been also shown that neural stem cells can be made to produce various blood cell types when implanted into the hematopoietic system (Bjornson, Rietze, Reynolds, Magli, & Vescovi, 1999). This illustrates that the neurovascular relationship is very closely associated, to the point that cells from one system can substitute for cells of the other.

Along with these observations, it is also known that both neurons and blood vessels share many of the same molecular cues during development. For instance Semaphorins, once thought to be involved only with axon guidance, have been shown to play a role in the developing vascular system as well. Proteins that are crucial in synaptic function have been found to be expressed by ECs as well. The expression of neurexin and neuroligins in endothelial and smooth muscle show the close relationship between the two systems (Arese et al., 2011). This interdependence of neuronal and vascular systems illustrates the possible importance of promoting re-vascualrization after SCI. If done correctly, promoting angiogenesis into an area of damaged spinal cord may facilitate the infiltration and establishment of new axonal connections.

Creating a bridge

There are many physical obstacles which must be overcome in order to effectively repair a SCI. With contusional SCI, cell death via liquefactive necrosis along with the formation of the gliotic scar, result in a fluid filled cyst. This cyst creates a physical gap which regenerating axons and vessels cannot traverse. In order to overcome this obstacle these physical barriers must be addressed. One approach is to utilize a structure which contributes to axonal regeneration as well as angiogenesis. Many approaches have been explored by other research groups to bridge this gap using various types of scaffolds and bridging materials.

There has been some success in promoting cellular growth into a graft (Cholas et al., 2012), (Bunge, 2008), but very few have successfully coaxed axons from one side of the gap to the other. Even more importantly, there seems to be an axon centric view of regeneration with these models, with little if any thought given to the establishment of a vascular supply. In order for a bridging strategy to be fruitful, the bridge has to have several characteristics: the bridge must be biocompatible, elicit little if any immune response, direct the growth of axons, and activate an angiogenic response. The bridge must have a solid 3D structure, but be permeable enough for cellular infiltration. The method used in our lab, air gap electrospinning, is a method which can fulfill all of these criteria. Illustrated in Figure 1.5, the spinning apparatus allows us to create a 3D structure with high tensile strength and porosity. This technique allows the grouping 80-90 million longitudinally aligned fibers in a 1mm diameter implant. The material used to spin the fibers is known as Polydioxanone Suture (PDS), which is an FDA approved absorbable suture. It is biocompatible compound that can persist *in vivo* for up to six

months. The fibers created using this process have shown the ability to not only direct axon growth, but elicit an angiogenic response as well (Chow et. al, 2009; Lin, 2010).

In this particular study, we will be focused on the potential of the implant to evoke an angiogenic response. It has been shown in previous studies in our lab that endothelial cells tend to associate with monofilaments (Lin, 2010). By controlling the concentration of PDS used in the electrospinning process, we can effectively control the overall size of the fibers present within the implant. Lower concentrations produce a predominance of smaller diameter fibers, and higher concentrations produce fiber with larger overall diameters. We hypothesize that ECs will infiltrate scaffolds with smaller diameter fibers to a greater degree than scaffolds with larger diameter fibers. Scaffolds with smaller fibers produce greater overall surface area for EC's, as well as other essential cell types, to use for migration into the lesion area. This will lead to a greater angiogenic response than scaffolds containing larger fibers.

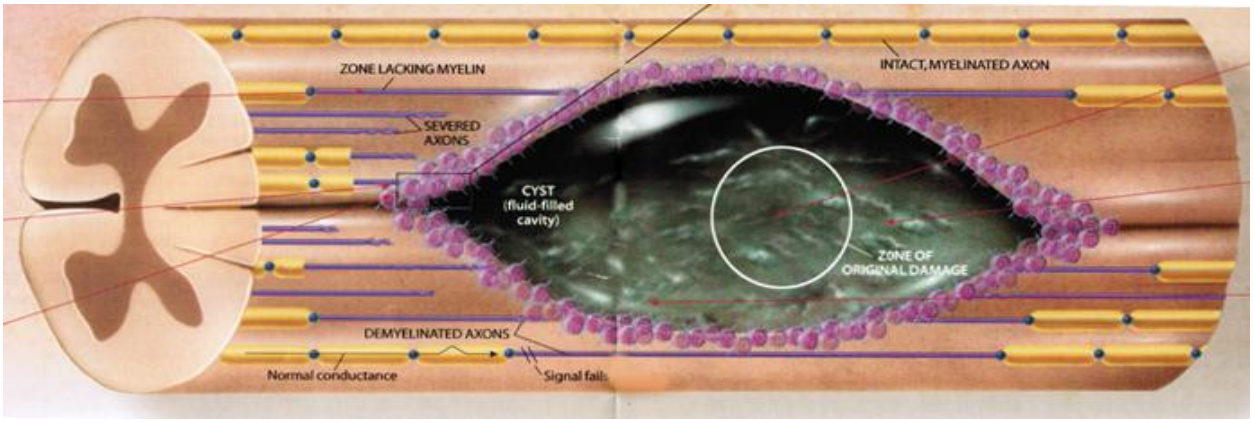
ZONE LACKING MYELIN
INTACT, MYELINATED AXON
SEVERED AXONS
CYST (fluid-filled cavity)
ZONE OF ORIGINAL DAMAGE
DEMYELINATED AXONS
Normal conductance
Signal fails

Figure 1.1 Fluid Filled Cyst. An illustration representing the fluid filled cavity and the surrounding glial scar which forms following SCI. This image was altered from McDonald et al. (1999).

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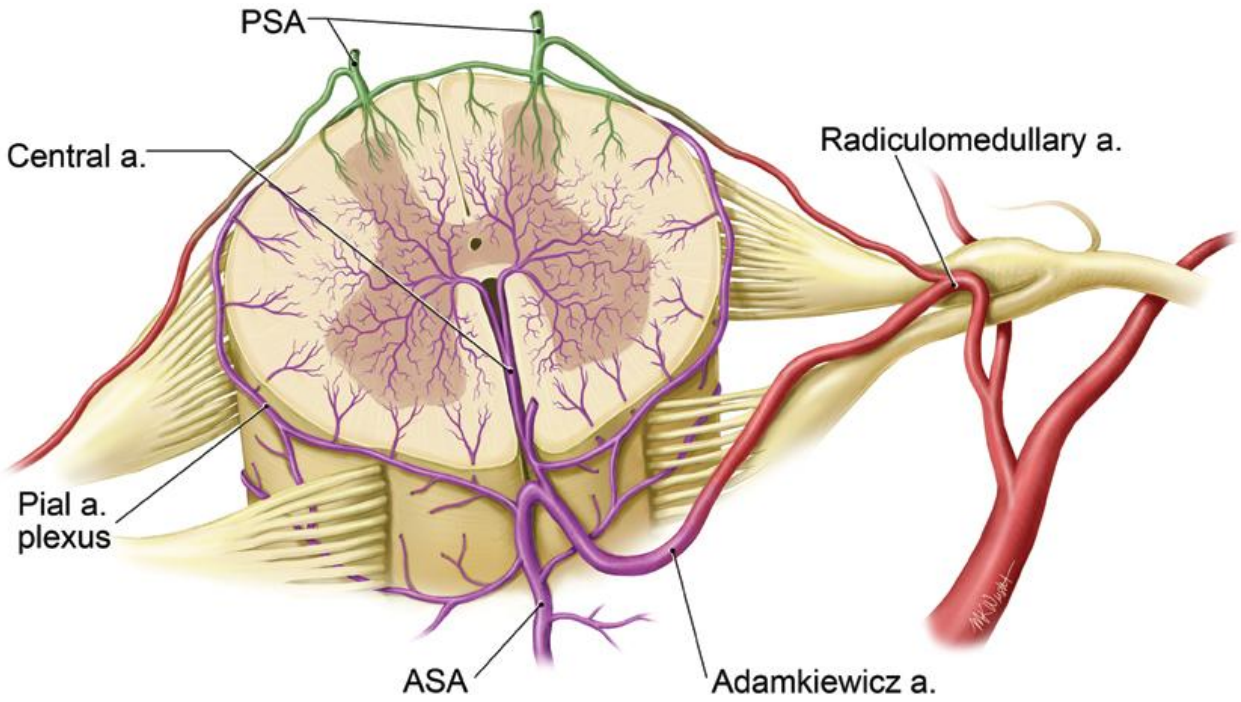
PSA
Central a.
Radiculomedullary a.
Pial a.
plexus
ASA
Adamkiewicz a.

Figure 1.2 Spinal Cord Blood Supply. This is an illustration showing the arterial blood supply to the spinal cord. This image was borrowed from Martirosyan et al (2011).

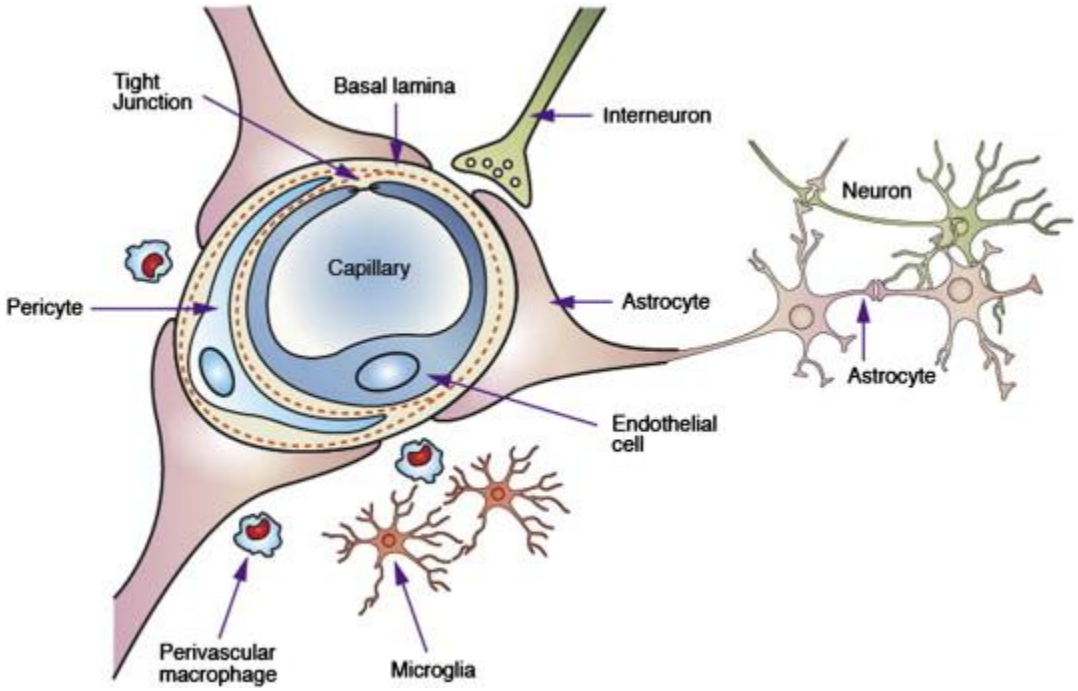
Tight Junction
Basal lamina
Interneuron
Neuron
Capillary
Pericyte
Astrocyte
Astrocyte
Endothelial cell
Perivascular macrophage
Microglia

Figure 1.3 The Neurovascular Unit. This is an illustration showing the cells which compose the neurovascular unit. This unit is what creates the Blood Brain/Spinal Cord Barrier. This image was borrowed from Chen and Liu (2012).

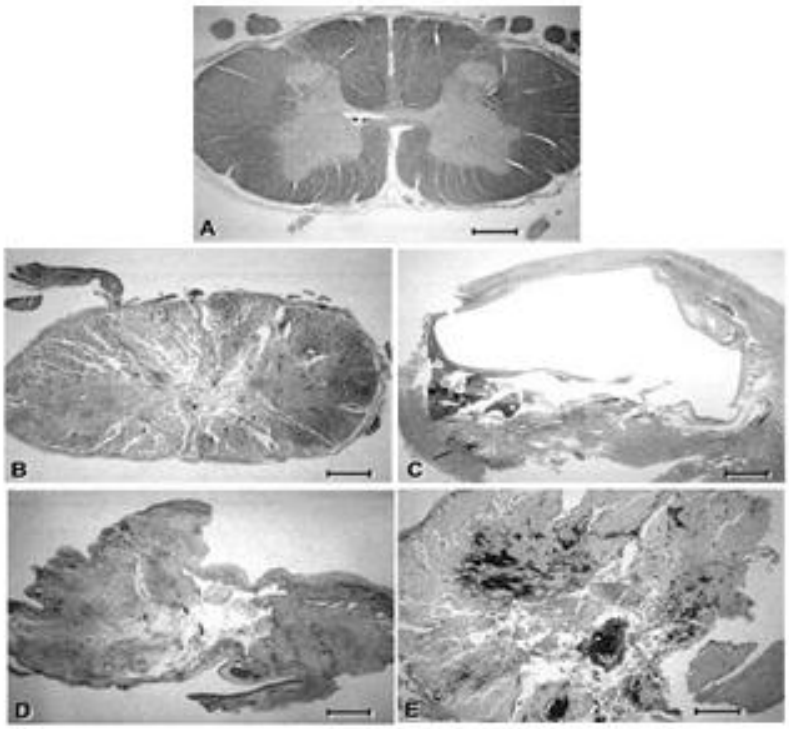
A
B
C
D
E

Figure 1.4 Spinal Cord Injury Pathology. (A) Cross section of a normal human spinal cord at the cervical level. (B) Solid cord injury with related loss of cellular architecture. (C) Contusion/cavitation injury with associated fluid filled cyst. (D) Laceration injury with pial disruption. (E) Massive compression injury showing tissue loss and hemmoraging. Scale bar for images A-E represent 2mm. (Norenberg et al., 2004)

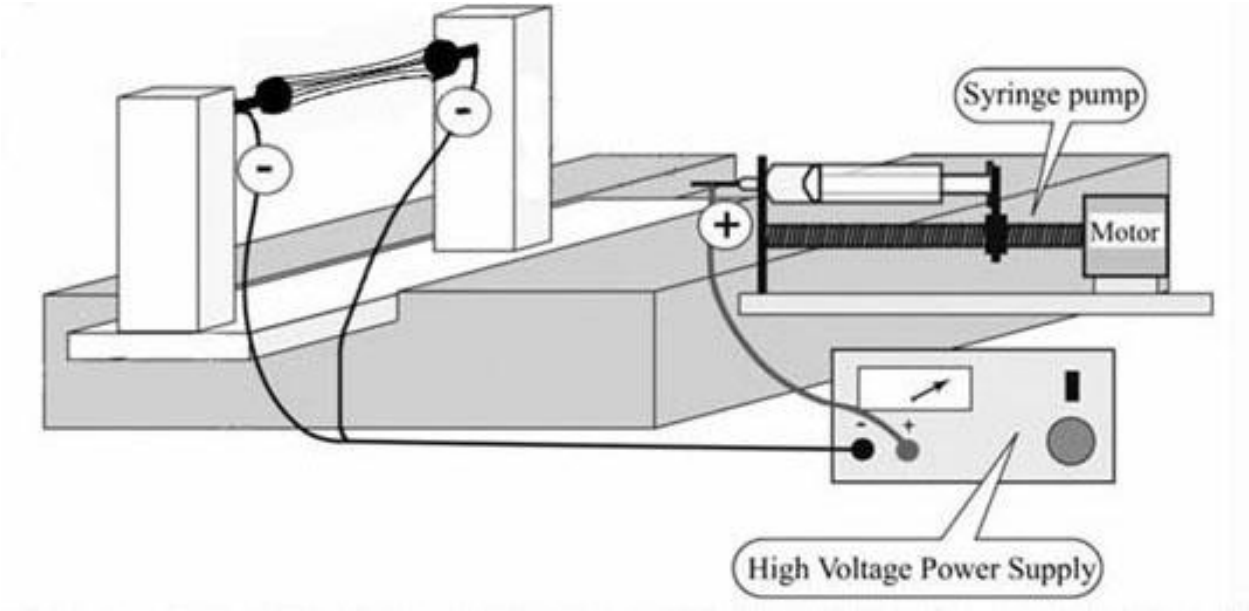
Syringe pump
Motor
High Voltage Power Supply

Figure 1.5 The Air-Gap Electrospinning Setup. This illustration shows the setup for Air-Gap Electrospinning. This system requires a high voltage DC power supply which connects to both the needle tip (18-guage, blunt end) and the two grounded poles between which the 3D scaffold is formed.

Chapter 2

Methods and Materials

Air Gap Electrospinning:

Polydioxanone Suture, or PDS (monofilament suture, Ethicon) is dissolved in 1,1,1,3,3,3 hexafluoro isopropanol (Sigma Aldrich) at a concentration of 100 mg/ml and another at 150 mg/ml. This solution is then ready to be electrospun, and is placed within a 5-ml syringe fitted with an 18-guage blunt end needle. The syringe is then placed into a syringe pump which allows for the precise control of disbursement of the PDS solution. The tip of the needle is attached to a high voltage, low amperage DC power supply (Spellman CZE1000R) by way of an alligator clip, and is charged to 22 kV. Two grounded horizontal poles separated by a 5-10cm gap composed of a two pole system which is used to capture the fibers as they are produced (Figure 1.5). This system results in the production of cylindrical monofilaments ranging in diameter from 1-12 μm. Theses fibers are collected between the two poles, and are arranged parallel to one another creating an overall cylindrical structure which becomes the core of the implant itself. The overall alignment of the monofilaments was determined by analyzing images taken on a scanning electron microscope (SEM). These photos were examined using Image J software and conducting a Fast Fourier Transformation, and it was determined that the monofilaments in both implants were highly aligned (Figure 4.2E).

Addition of the Outer PGLA capsule:

Upon completion of the core of the matrix via the airgap electrospinning, there is the addition of a secondary copolymer Polyglycolic-Lactic Acid (PGLA). PGLA consists of 50% polyglycolic acid and 50% polylactic acid, and is prepared in the same manner as the PDS mentioned earlier. The application of the PGLA is done by using a grounded plate in place of the two pole system. The matrix is placed on the grounded plate, and is moved to the appropriate distance from the charged needle. Upon application of the voltage, the PGLA copolymer is electrospun toward the grounded plate. The PGLA is allowed to accumulate onto the PDS matrix, with the fibers arranged perpendicular to the PDS fibers. The PDS matrix is periodically rotated to ensure it is evenly coated. This process effectively encapsulates the matrix and effectively holds the PDS monofilaments together. The capsule also helps to direct the flow of infiltrating cells into the matrix from either side rather than at any point along its length.

Animal Procurement and Treatment:

All animal work in this study was done following the VCU IACUC approved protocol. Animals were obtained from Harlan Inc. The rats used in this study were 100 day +/- 10 day old female Long Evans Hooded rats.

Animal groups

Six separate groups were used in this study to observe the progression of angiogenesis over time. An n-number of 3 was used in each group for a total of 18 rats. The groups that were examined received electrospun PDS scaffolds of 100mg/mL or 150 mg/mL at 1 week, 1 month, and 2 months.

Surgical Procedure:

The rats were anesthetized with 5% Isofluorane (AERANE) in a gas chamber initially, and then transferred to the surgical table and placed into a nose cone which delivered 3% Isofluorane throughout the duration of the surgery. The animal was placed onto a warming blanket and a rectal temperature probe was inserted to monitor the animal's core temperature. The back of the animal was then shaved and the skin treated with Betadine to ensure an aseptic surgical site. A dorsal midline incision was made using a #10 blade scalpel to expose the muscles of the back. Two additional incisions were made along either side of the spinous processes, and tissue spreaders were inserted underneath incised muscle. The tissue spreaders were then opened in order to expose and allow access to the vertebrae. The location of the T9 vertebrae was identified via palpation of the back. The spinous process was then removed using a pair of Rongeur's, allowing access to the space between T9 and T10. Rostral portions of the T10 lamina were removed in order to create a wider space between the vertebrae, and the spinal cord was then transected using a pair of micro scissors. A 1mm section of the electrospun matrix (either 100 mg/ml or 150mg/ml depending on group) was then inserted into the lesion site. Upon completion of matrix insertion, the tissue spreaders were then removed and the muscular incisions were close using silk suture (Ethicon). The skin was then stapled closed using Mitchell staples (FST). An antibacterial ointment (Bacitril) was applied to the incision site to prevent infection.

Post Operative Care:

Upon completion of surgery, rats were placed onto a heating pad and monitored until restoration of consciousness. Rats were then given IP injections of Gentamicin (10mg/kg) and Buprenorphine (0.5 mg/kg). Buprenorphine was administered in 12 hour intervals for the first 48 hours post operation. Rats were also given injections of Gentimycin for 7 days post operation to prevent bacterial infection. Acetaminophen (150 mg/kg) was administered via addition to drinking water for 3 days post operation. Bladders were voided of urine manually every 8 hours until the rats regained bladder function. Upon diagnosis of bladder infection, rats were treated with 5 day cycles of Gentimycin.

Assessing Functional Recovery: Basso-Beattie-Breshnahan Locomotor Rating Scale:

The BBB locomotor rating scale is a well known and widely used behavioral test to quantify animal behavior and functional recovery. The rats are scored on a 21 point scale according to the amount of hind limb motion observed. The scale ranges from a 0-21; 0 being complete hind limb paralysis, and 21 being fully functional hind limb mobility. The test assigns points in relation to number of joints involved, coordinated movement of limbs, weight bearing ability, and gait stability. The rats were first evaluated pre-operatively for 4 minutes in an open field to ensure all animals began at a score of 21, indicating no pre-existing motor deficiencies. To assess the functional recovery post operatively, the animals were placed into an open field (a large round top table with a clear plexiglass wall enclosing the animal) and allowed to move freely about the space for a 4 minute period. Animals were monitored by two separate viewers, and a score

was assigned based on the observed movement in relation to the scale. The animals were evaluated on a weekly basis for the duration of the experiment (1- 8 weeks).

Tissue Collection:

All rats were euthanized using a pentobarbitol agent Euthasol (150 mg/kg) injected intraperitoneally to induce anesthesia and were transcardially perfused with 0.1 M phosphate-buffered saline (PBS). Animals were then fixed using 4% paraformaldehyde for immunohistochemistry, or a 4% paraformaldehyde and 1% glutaraldehyde mixture if the tissue was to be processed for electron microscopy (EM). The spinal cords were then dissected out of the spinal columns and place into conical flasks containing paraformaldehyde or paraformaldehyde/glutaraldehyde mixtures for 48 hours at 4 degrees Celsius. Spinal cords which to be sectioned for immunohistochemistry were then transferred to a 30% sucrose solution for 24 hours at 4 degrees Celsius. 40μm thick longitudinal and coronal sections of the implant site and bordering spinal tissue were cut using a cryostat.

Immunohistochemistry:

The antibody Rat Endothelial Cell Antigen-1(RECA-1) was used to identify newly formed vasculature within the injury site. RECA-1 labels endothelial cells which allows for the visualization of the vasculature when combined with a fluorescent-conjugated secondary antibody. All sections were blocked with 10% normal goat serum (NGS) in 0.1M PBS. Sections were then washed in PBS and incubated with primary antibody (RECA-1) (1:100) in 1.5% NGS in PBS overnight at 4 degrees Celsius. Sections were then washed again in PBS and then incubated with Alexa Fluor 598 (1:400) fluorescent

conjugated secondary antibodies (Invitrogen). Sections were then stained using Bisbenzimide (1:1000), an intercalating agent which labels cell nuclei by inserting itself in between Adenine-Thymine base pairs. Slides were then washed 3 times in PBS. Sections were then immersed in mounting media (Vectashield) and coverslipped. The presence of RECA-1 positive vessels was determined using a Leica confocal moicroscope. The number of vessels present per section was determined using IP Lab software. Confocal images of coronal sections were opened in IP lab, and vessels were counted by outlining each RECA-1 positive vessel resulting in an overall vessel count per section.

Transmission Electron microscopy:

Processing for EM began with implantation site being post-fixed in buffer gluaraldehyde and then in 1% OsO4 for 1 hour. The section was then dehydrated through an ethanol series and then infiltrated overnight in epon/araldite. Semi-thin (1µm) coronal sections were obtained and stained with toluidine blue. These sections were used to affirm that the correct portion of the lesion area was exposed and ready to be cut into thin sections (500 nm) for EM. Monofillament diameters were measured by taking representative grids though the middle of the lesion/implant site using a Joel JEM-1230 transmission electron microscope.

Scanning Electron Microscopy

Imaging of 100mg/mL and 150mg/mL implants was done using a Zeiss EVO 50 XVP scanning electron microscope. Samples of matrix were sputter coated with gold and

inserted into the microscope for visualization. Monofilament diameters were measured using SmartSEM visualization software.

Chapter 3

Histological Study of the Vascular Supply in the Rat Spinal Cord

Introduction:

Following a spinal cord injury, there is a disruption to the normal vascular architecture. The vascular supply is fundamental to the growth, function and survival of all of the cells within the spinal cord as well as the rest of the body. Vessels infiltrating the tissue are responsible for providing oxygen and other trophic factors as well as removing cellular waste products. Inflammation and other secondary mechanisms, including gliotic scar formation, and the loss of vascular function will prevent the regeneration of tissue in the damaged areas. Following SCI, it is imperative to re-establish a vascular network in order to provide an environment conducive to regeneration. In this chapter we will examine the distribution of vessels within normal spinal cord tissue. We will also explore our novel method for inducing an angiogenic response by utilizing an electrospun matrix.

Vessel Distribution in the Spinal Cord

The vascular supply in the spinal cord has a very distinct pattern that is apparent when examined through histological means. As we look at a coronal section of the normal rat thoracic spinal cord (Figure 3.1A), we can see clear delineation of the white and gray matter. The characteristic butterfly pattern in the center of the section marks

the gray matter; an area composed of neuronal cell bodies and other associated support cells. The area surrounding the gray matter is the white matter where the various axonal tracts are located.

Utilizing an antibody known as Rat Endothelial Cell Antigen-1 (RECA-1), it is possible to visualize the vascular network and the relative distribution of blood vessels. The majority of the vessels within the cord are located within the gray matter. This dense network of vessels is needed to support the high metabolic demand of the neuronal cell bodies which occupy this area. The vascular supply found within the white matter, the axonal bearing area of the spinal cord, is much less dense and is distributed over a wider area. The vessels radiate inward from the surrounding pial arterial plexus toward the core, and are distributed in a radial fashion (Figure 3.2A). A closer comparison of the white and gray matter shows a clear difference in blood vessel distribution (Figure 3.2B).

Angiogenesis and the Scaffold

One of the major hurdles to overcoming SCI and promoting functional recovery is the reestablishment of the vascular supply. In order to recreate an environment similar to the normal tissue, it is imperative to induce angiogenesis in a way that mimics the original vascular architecture. In this study we wanted to further explore the ability of the scaffold to induce an angiogenic response, and to compare vessel density and distribution within the matrix to that of normal tissue.

As the electrospinning process in our lab has evolved, it has been demonstrated that scaffolds with monofilaments that are highly aligned allow for greater cellular

infiltration into the graft, as opposed to matricies with randomly oriented fibers (Figure 3.3). Other studies utilizing the aligned matrix have shown that in some instances infiltrating ECs were interacting with the monofilaments as they entered the scaffold (Figure 3.4A). It was hypothesized that the ECs were using monofilaments as a template for growth and infiltration. The vessels began to grow and increase their luminal size, and began to disassociate from the monofilaments (Figure 3.4B, C). Ultimately these newly formed vessels began functioning, confirmed by the presence of circulating red blood cells (Figure 3.4D, E).

The use of the aligned scaffold in this study was preferred due to the ease of cellular infiltration, as well as the ability of monofilaments to interact with incoming endothelial cells as described. It was also thought that due to the highly aligned nature of the fibers in the scaffold, the in growth of blood vessels from the rostral and caudal ends of the lesion could achieve a more direct path through the scaffold. By virtue of this spatial arrangement, this directed growth may allow tissue to maintain the same plane as is seen in the normal spinal cord. For instance, blood vessels in the dorsal columns rostral to the lesion would be connected by monofilaments to vessels on the caudal side. By maintaining this spatial relationship, it may be possible to facilitate faster regeneration and anastamosis of vessels on both sides of the lesion.

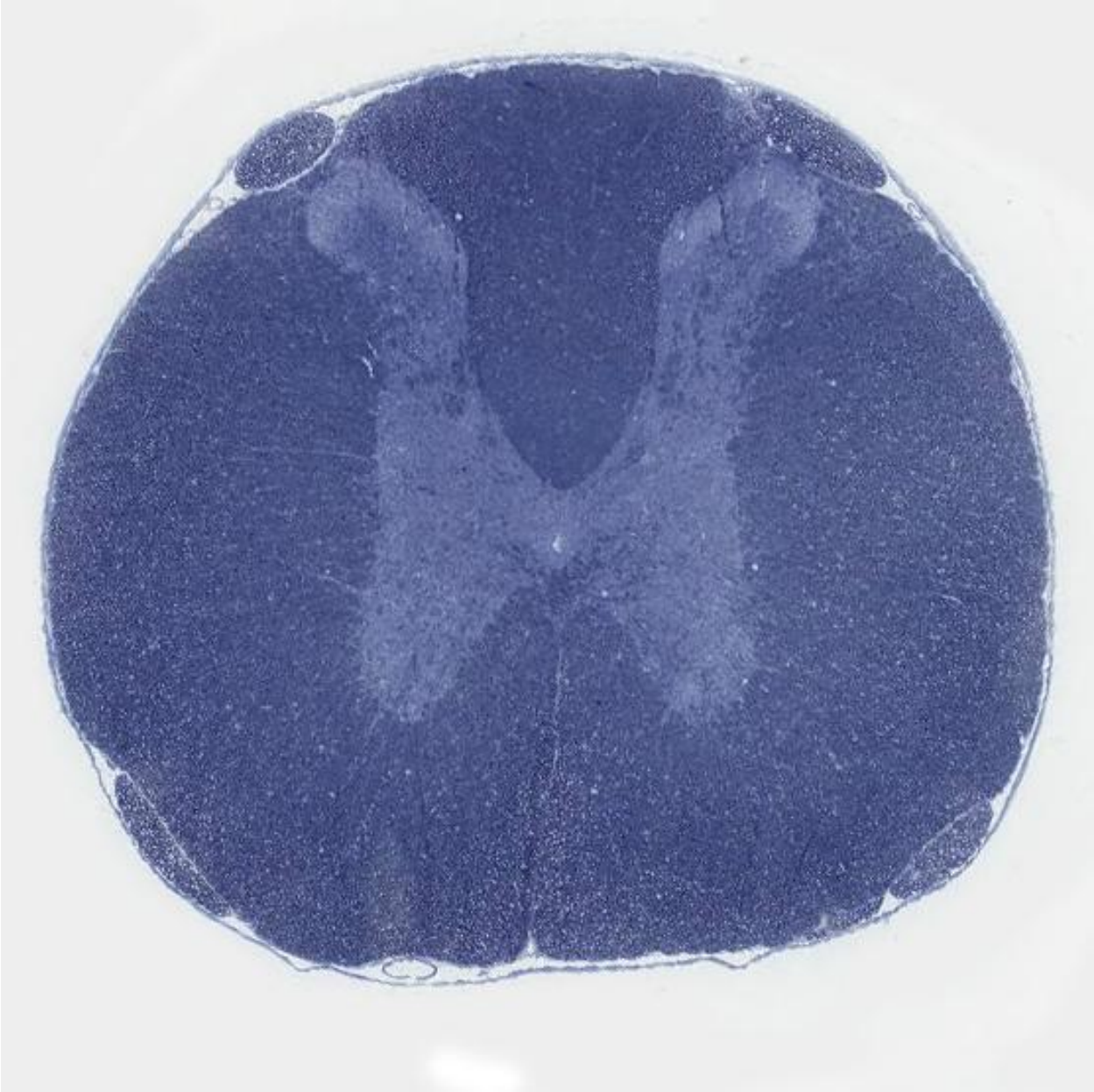

Figure 3.1 Normal Rat Spinal Cord. A cross section of a normal rat spinal cord at the thoracic level showing the delineation of white matter versus gray matter. The section is oriented with the dorsal end at the top of the image.

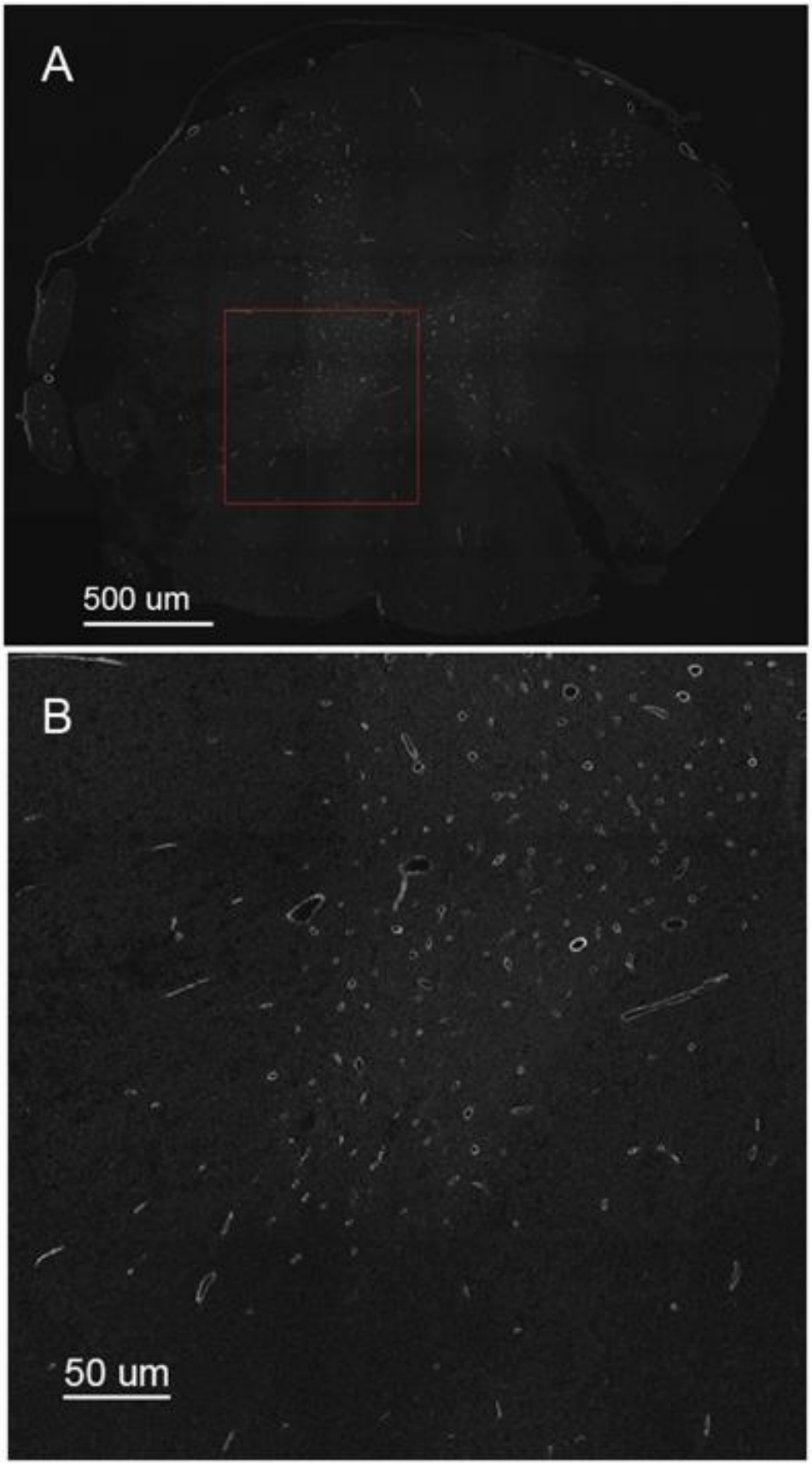
A
500 um
B
50 um

Figure 3.2 Vasculature in the Normal Thoracic Spinal Cord. (A) Cross section of a normal rat spinal cord stained with RECA-1showing blood vessel distribution in white vs. gray matter. (B) Close up of blood vessels in white versus gray matter showing greater overall density in the gray matter.

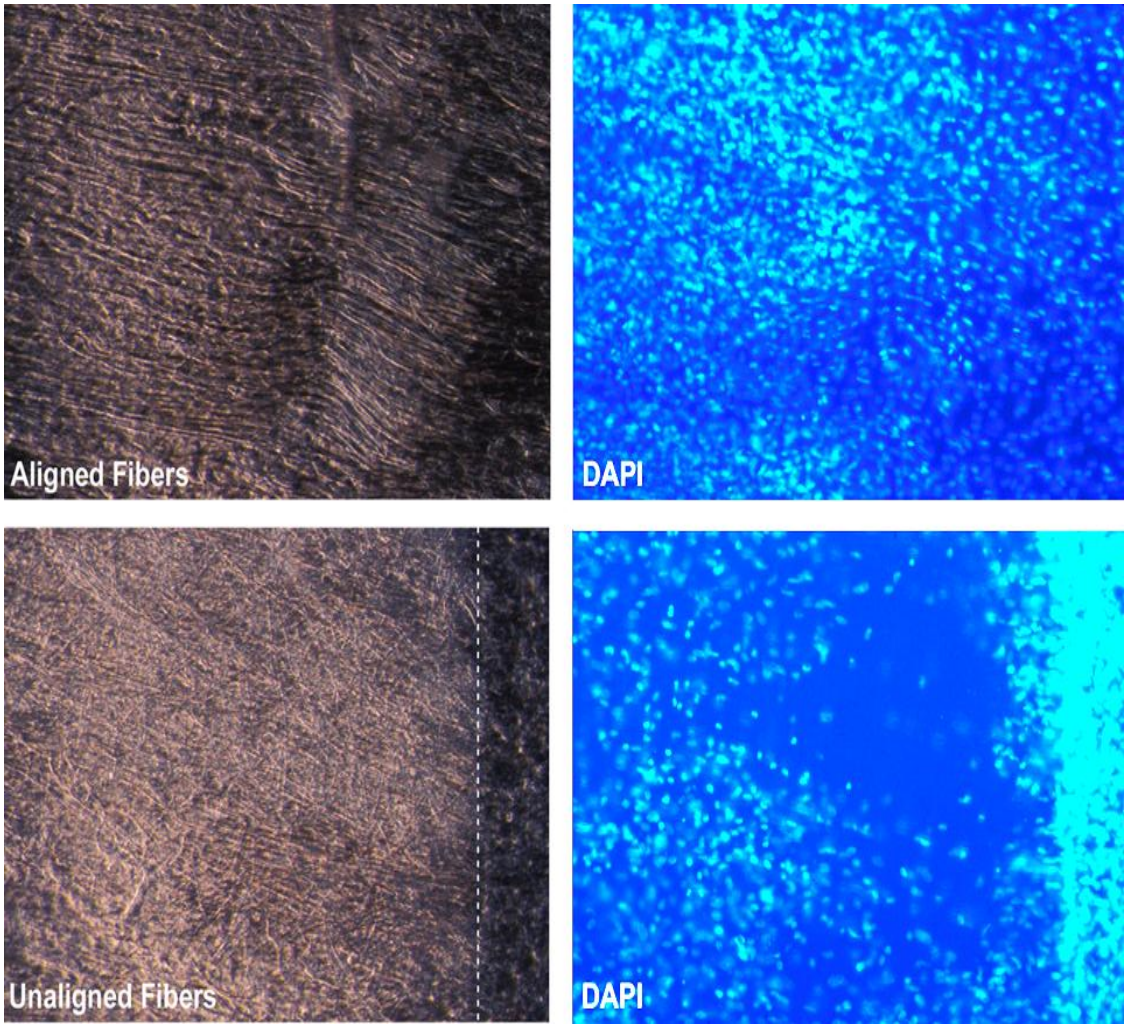
Aligned Fibers
DAPI
Unaligned Fibers
DAPI

Figure 3.3 Cellular Infiltration. DIC images of aligned versus unaligned fibers. Corresponding cellular infiltration is shown through DAPI staining of cell nuclei. The cells in the implant with aligned fibers are greater in number and more evenly distributed than those in the unaligned implant.

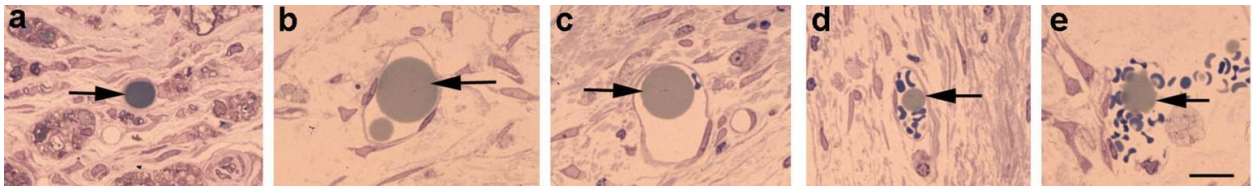
a
b
c
d
e

Figure 3.4 Monofilament-Endothelial cell Interaction. (A) LM photograph of endothelial cell tightly associated with a monofilament. (B, C) LM photograph showing ECs beginning to dissociate from the monofilaments. (D, E) LM photograph showing vessel lumens containing monofilaments and circulating red blood cells.

Chapter 4

Generation and Testing of Bridges with Varying Monofilament Diameters

Introduction:

Spinal cord injuries present many obstacles that must be overcome in order to facilitate functional recovery. Most often the axons which carry sensory and motor information to and from the brain are damaged or severed, leaving an individual with varying losses of function. The secondary injuries to the damaged areas, which include the formation of a fluid filled cyst and the creation of the gliotic scar, inhibit axonal regeneration and revascularization. A novel therapeutic strategy is required to overcome these physical limitations and create an environment in which cells can survive. Our aim is to address these problems by using a biologically compatible structure on which regenerating axons and infiltrating endothelial cells can grow. To do this we utilized air-gap electrospinning in combination with a biocompatible material; PDS, an type of suture that breaks down naturally in the body over a certain course of time.

We have shown in previous experiments that our electrospun scaffolds serve exactly this function. One experiment shows that the highly aligned fibers in the scaffolds direct the outgrowth of nurites in the same direction and orientation of the monofilaments (Figure 4.1). Another experiment conducted in our lab demonstrated the

monofilaments interacting directly with infiltrating blood vessels. The Endothelial cells which compose these vessels are seen wrapping monofilaments, and over the course of time the filament breaks down resulting in a lumen. In some instances vessel lumens can be observed containing deteriorating monofilaments accompanied by red blood cells, indicating the creation of a functioning blood vessel. Motivated by these observations, it is our hypothesis that endothelial cells may be attracted or influenced by monofilaments of a certain size. To test this hypothesis we created two separate implants, each implant having fibers within a specific range of diameters. One of the implants contains smaller diameter fibers, and the other containing fibers with larger diameters overall.

Creating the Bridges

Two separate concentrations, 100 mg/mL and 150 mg/mL, of PDS were used to create scaffolds with different fiber diameters. The 100mg/ml concentration created a scaffold with fibers that were between 1- 1.8 micrometers in size, respectively. Concentrations of PDS at 150mg/mL yielded a scaffold with larger monofilaments in the 2- 2.8 micrometer range (Figure 4.2D). Images of the 100mg/mL and the 150mg/mL implants were taken at the same magnification, and the fiber diameter measurements were made using Zeiss SmartSEM software. The difference in the fiber size can be seen readily when comparing SEM photos of fibers electrospun at different concentrations (Figure 4.2 A, B, C). Monofilaments within the scaffolds need to be highly aligned in order to give the straightest possible route for incoming cells into the lesion area. Using ImageJ software, a calculation known as a Fast Fouier Transfom (FFT) was performed to determine the overall alignment of the monofilaments. The FFT

confirmed that our scaffolds have significant alignment in the desired direction as indicated by the graph (Figure 4.2 E).

Results:

For this experiment, a full transection was performed at thoracic level 9. Using this particular type of injury allows for easy insertion of the electrospun scaffolds. The two implant types were inserted into animals, and animals were allowed to survive for three separate time periods; 1 week, 1 month, or 2 months. These time points correspond to two of the various phases of recovery following spinal cord injury. The first phase is referred to as acute, and takes place seconds to minutes after the initial insult. The next phase is referred to as secondary, and takes place minutes to weeks after an injury. The final phase is known as chronic, and can take place over the course of months to years after injury. These three separate time points allow us to analyze how vascularization is influenced by the two separate scaffolds during the secondary and chronic phases, and how this may aid in mitigating secondary injury progression and promoting regeneration.

Histology

The analysis of the vessel distribution was examined by removing the spinal cord, and then sectioning the implanted site coronally. 40um thick sections from the center of the site were then stained with RECA-1 to label the vasculature. The RECA-1 positive vessels in each section were counted using IP lab software. The overall vessel numbers in the 100 vs.150mg/mL groups for each time point were compared to the number of vessels in a normal thoracic spinal cord section. The total number of vessels

counted in each experimental animal was normalized to the number of vessels found at a comparable spinal cord level in an uninjured control animal (Figure 4.3).

A large degree of variance was observed over each time point between each experimental group when normalized to an uninjured control animal, but the 100 groups consistently had a greater number of vessels than the 150 groups. This suggests that the scaffolds containing smaller diameter fibers induce a greater angiogenic response than scaffolds containing larger diameter fibers.

An interesting progression of angiogenesis can be seen when sections of the two scaffold types are compared. In the one week groups, both the 100 (Figure 4.5 A,B) and 150mg/mL (Figure 4.5 C,D) implants showed the presence of infiltrating blood vessels. When the vessels were counted, the 100mg/mL implant contained 242 individual vessels as oppsed to the 150mg/mL which contained 148.

The 1 month groups showed a further increase in blood vessel formation. The 100mg/mL group contained a significantly larger number of vessels (690), as opposed to the 150 mg/mL group (422). The vessels in the 100 group were smaller in diameter and more tightly packed together (Figure 4.6 B), while the 150 group showed vessels with larger diameters that were spaced farther apart (Figure 4.6D). The tissue in the100 group was also observed to have better overall continuity than the 150 (Figure 4.6A). The absence of vessels and tissue in the center of the in the 150mg/mL implant area suggests the beginning of cystic cavity formation (Figure 4.6C).

When examining the 100 versus the 150mg/mL sections in the two month group, we see that the 100 group produces better results than the 150. The number of vessels

in both of the 2 month groups decreased from the levels found in the 1 month groups; the section from the100 group contained 410 vessels, whereas the section from the150 group contained 264. The 100mg/mL section shows vessels that are evenly distributed, as well as having similar diameters (Figure 4.7 B). Vessels in the 150 group vary widely in size and distribution, and very little tissue is left intact (Figure 4.7 D). Both the 100 and 150 groups show the development of cystic cavities (Figure 4.7 A,C), but the difference in cavity size and tissue sparing is quite dramatic. The 100mg/mL section shows the development of a cystic cavity, but the 150mg/mL section has developed a cavity which occupies nearly the entire section.

One problem that required some further attention was the presence of holes or cavities within the individual sections. While the normalized vessel counts (Figure 4.3) compared the total number of vessels in each group to that of the control animal, in order to determine the vessel density, or vessels per unit area, the holes in the tissue had to be accounted for. Once the actual tissue area was determined by subtracting out the empty spaces, the percent of tissue occupied by vessels was able to be determined.

The percent of tissue occupied by vessels at each time point within each group was examined (Figure 4.4), and again we see that the 100 mg/mL group had a higher percentage of vessels occupying the tissue than the 150 mg/mL group. However, at the 2 month time point, the two groups have very similar percentages, with the 100 mg/mL group being only slightly higher than the 150 mg/mL group, but the amount of tissue remaining in the 150 mg/mL group is two thirds that of the 100 mg/ml group.

The differences in the number and distribution of blood vessels in the 100 vs. the 150mg/mL groups are quite remarkable. Not only does the 100 group stimulate the production of more vessels than the 150, but the development of the cystic cavity appears to be greatly reduced in the 100 group as well. These observations suggest that scaffolds electrospun at 100mg/ml of PDS are superior in terms of decreasing secondary injury progression as well as promoting a greater angiogenic response.

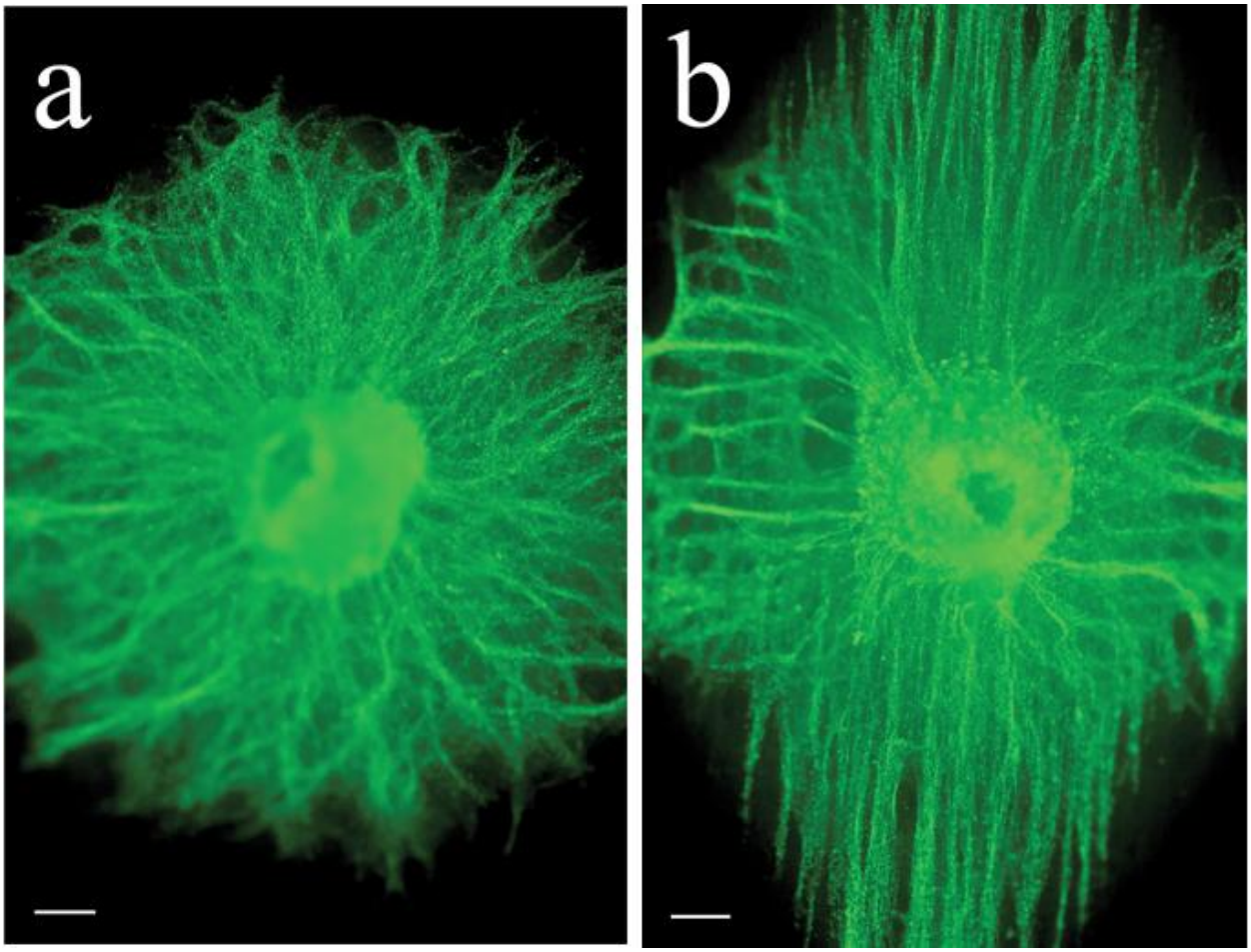
a
b

Figure 4.1 Neurite Growth. (A) Neurite outgrowth from a DRG on an unaligned electrospun matrix radiates outward in a randomized fashion. (B) Neurite outgrowth from a DRG on an aligned matrix is oriented in the direction of the fiber alignment, illustrating the aligned fibers ability to direct cellular growth. The neurites were stained using Tuj1. The scale bar represents 200um. This image was borrowed from Chow et al (2009).

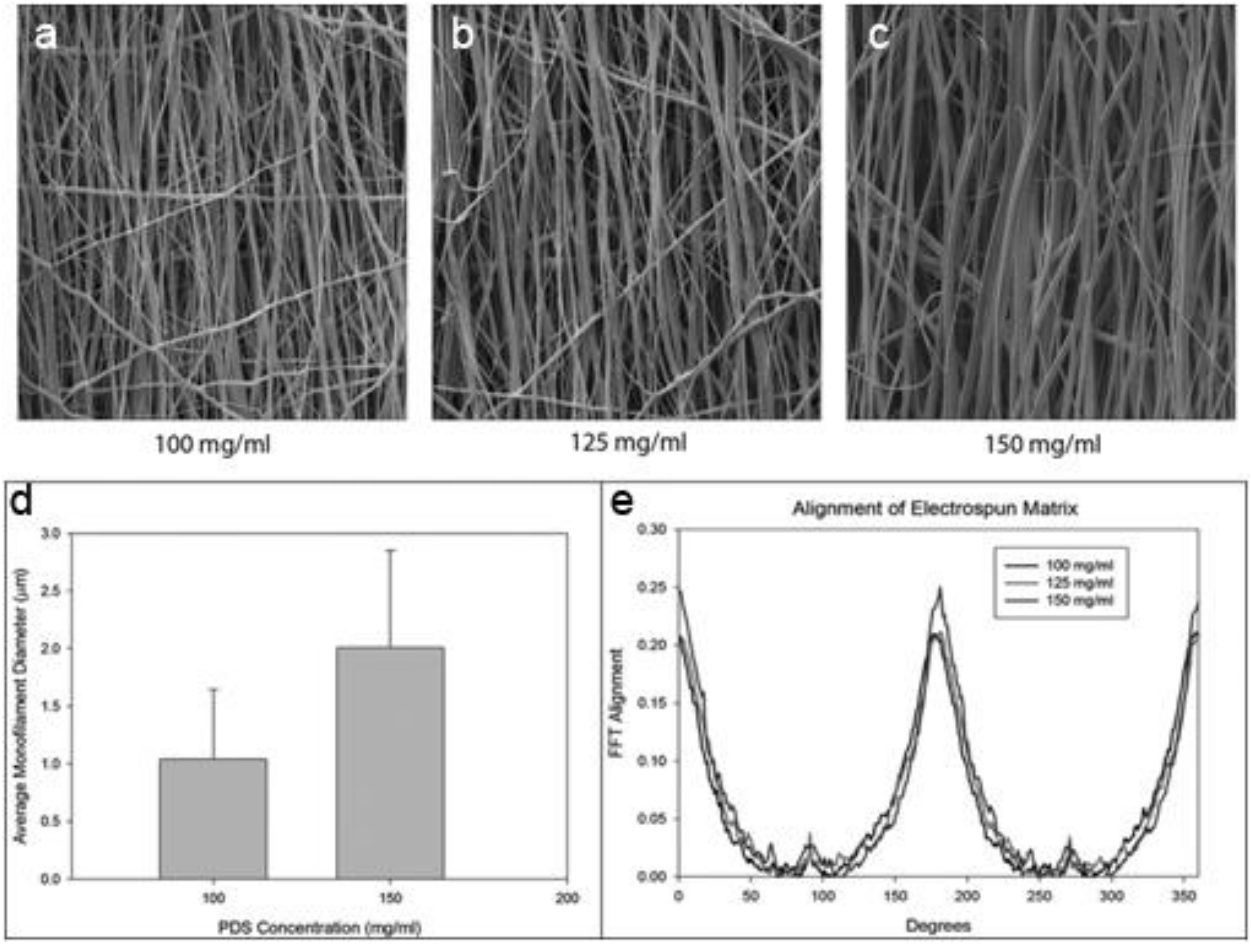
a
b
c
100 mg/ml
125 mg/ml
150 mg/ml
d
Average Monofilament Diameter (µm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
150
200
PDS Concentration (mg/ml)
e
Alignment of Electrospun Matrix
100 mg/ml
125 mg/ml
150 mg/ml
FFT Alignment
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
350
Degrees

Figure 4.2 Fiber Size and Orientation. (A,B,C) SEM images showing an increase in fiber diameters as the concentration of PDS is increased. (D) Graph comparing the range of fiber diameters in 100mg/mL versus 150mg/mL electrospun scaffolds. (E) An FFT confirming the majority of the fibers at varying concentrations are highly aligned.

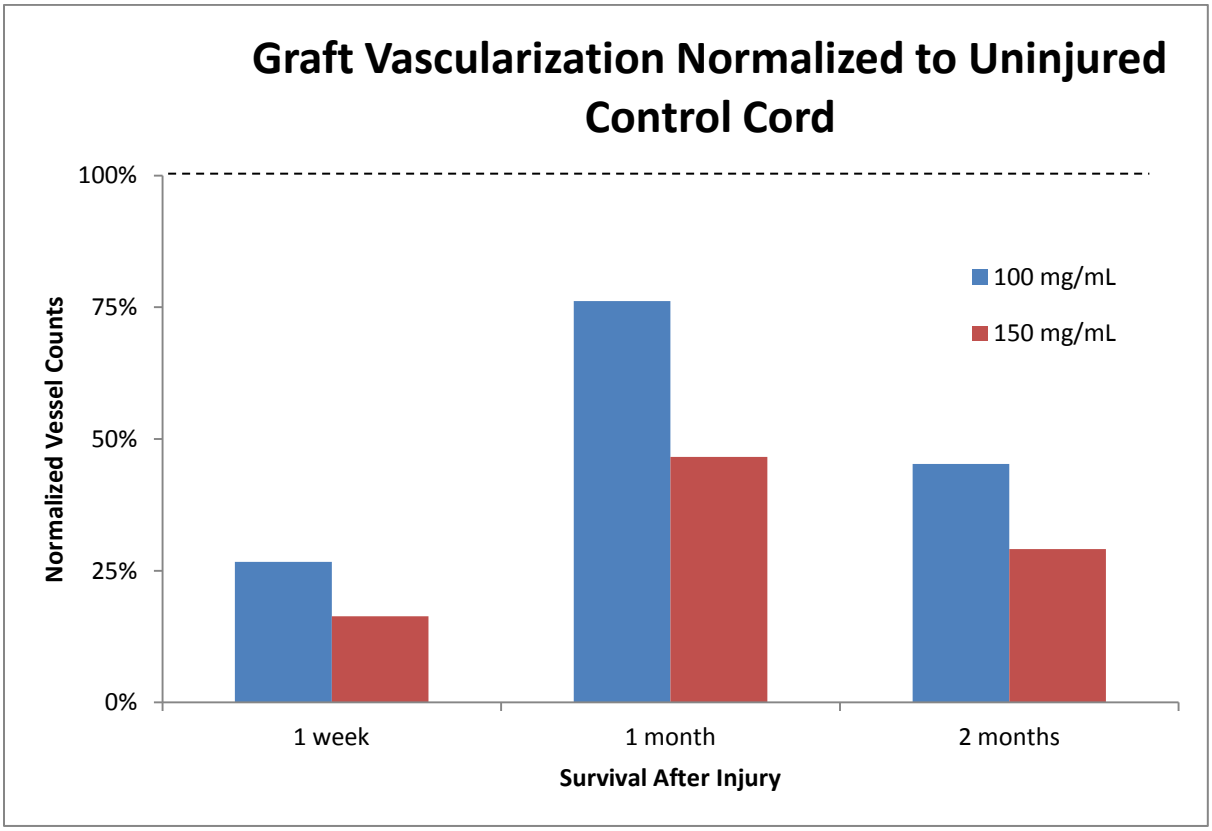
Graft Vascularization Normalized to Uninjured Control Cord
100%
75%
50%
25%
0%
Normalized Vessel Counts
100 mg/mL
150 mg/mL
1 week
1 month
2 months
Survival After Injury

Figure 4.3 Scaffold Vascularization Percentages. Bar graph showing normalized vessel counts at the three separate time periods. The 100mg/ml implants show greater vascularization than the 150 mg/mL implants over all three experimental time points.

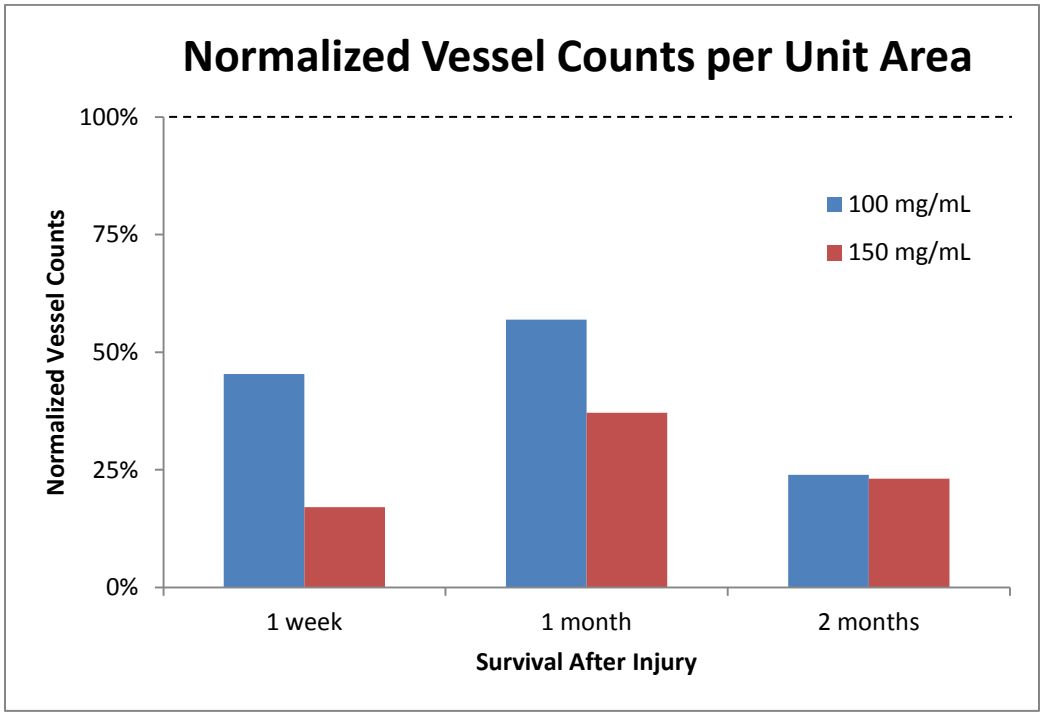
Normalized Vessel Counts per Unit Area
100%
75%
50%
25%
0%
Normalized Vessel Counts
100 mg/mL
150 mg/mL
1 week
1 month
2 months
Survival After Injury

Figure 4.4 Vessels Per Unit Area. Bar graphs showing normalized vessel counts per unit area. This graph has been adjusted to take into account the empty space that is found in the implant area.

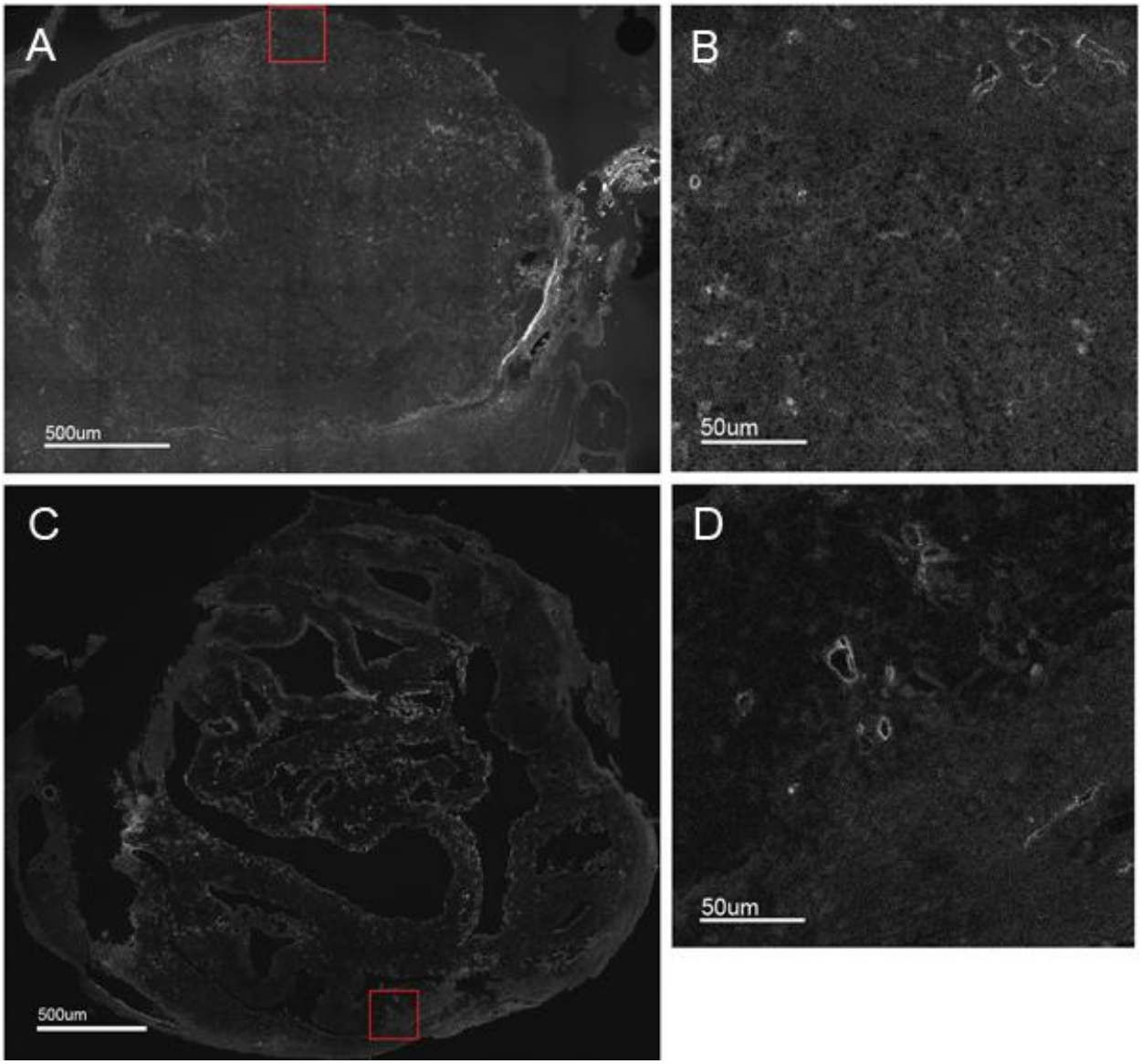
A
500um
B
50um
C
500um
D
50um

Figure 4.5 One Week Scaffolds. (A) Confocal image of a 100mg/mL implant from a one week animal. The fibers are smaller and pack tightly together, leaving few gaps in the implant. (B) A close up view of vessels outlined in panel A. (C) Confocal image of a 150mg/mL implant form a one week animal. The fibers have a larger diameter and may contribute to the layering effect creating more gaps. (D) A close up of the vessels outlined in panel C.

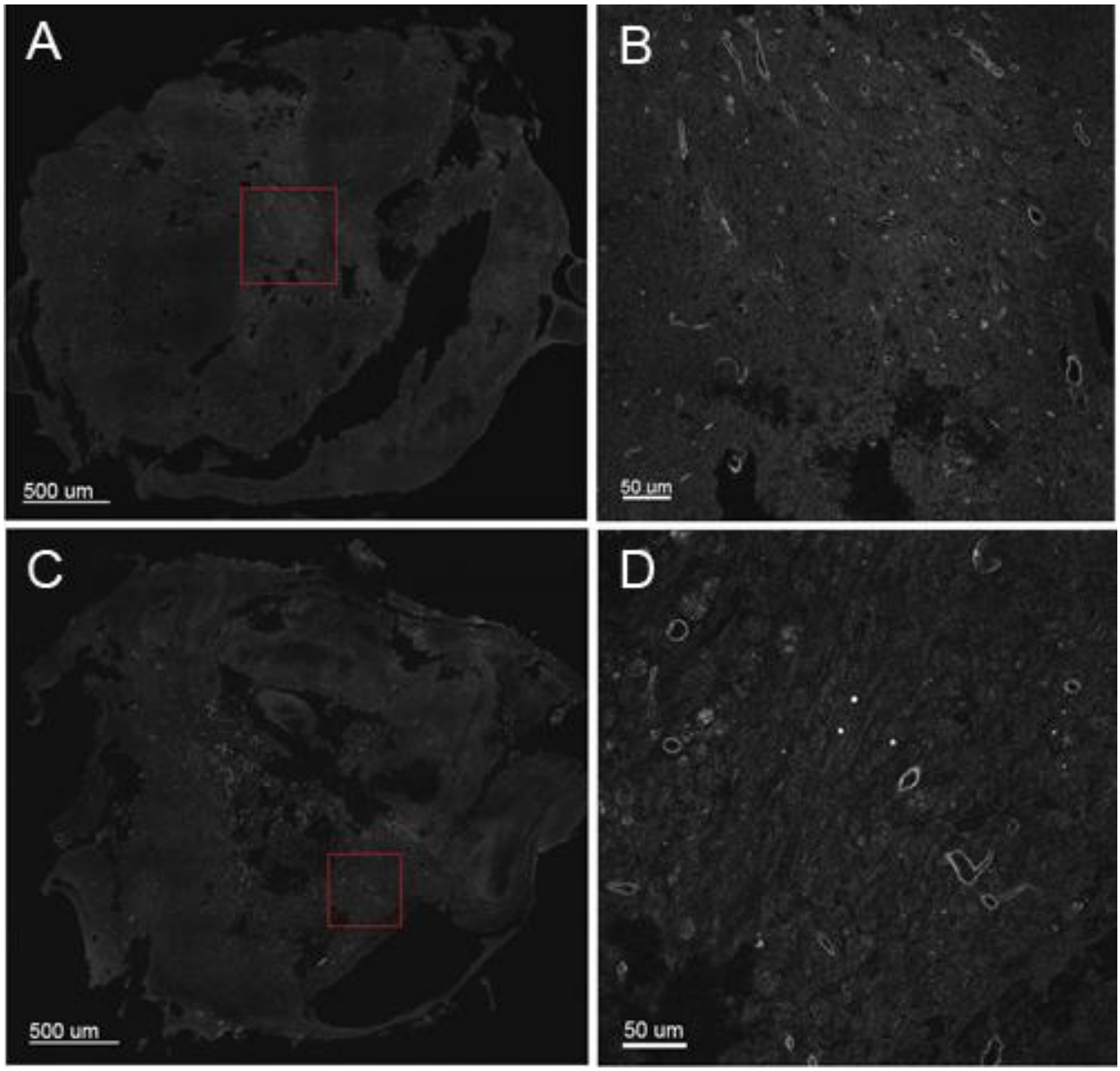
A
500 um
B
50 um
C
500 um
D
50 um

Figure 4.6 One Month Scaffolds.(A) Confocal image of a 100mg/mL implant from a one month animal. The tissue remains relatively intact. (B) A close up view of vessels outlined in panel A. (C) Confocal image of a 150mg/mL implant form a one month animal. The beginning of a cystic cavity can be seen in the center of the section. (D) A close up of the vessels outlined in panel C.

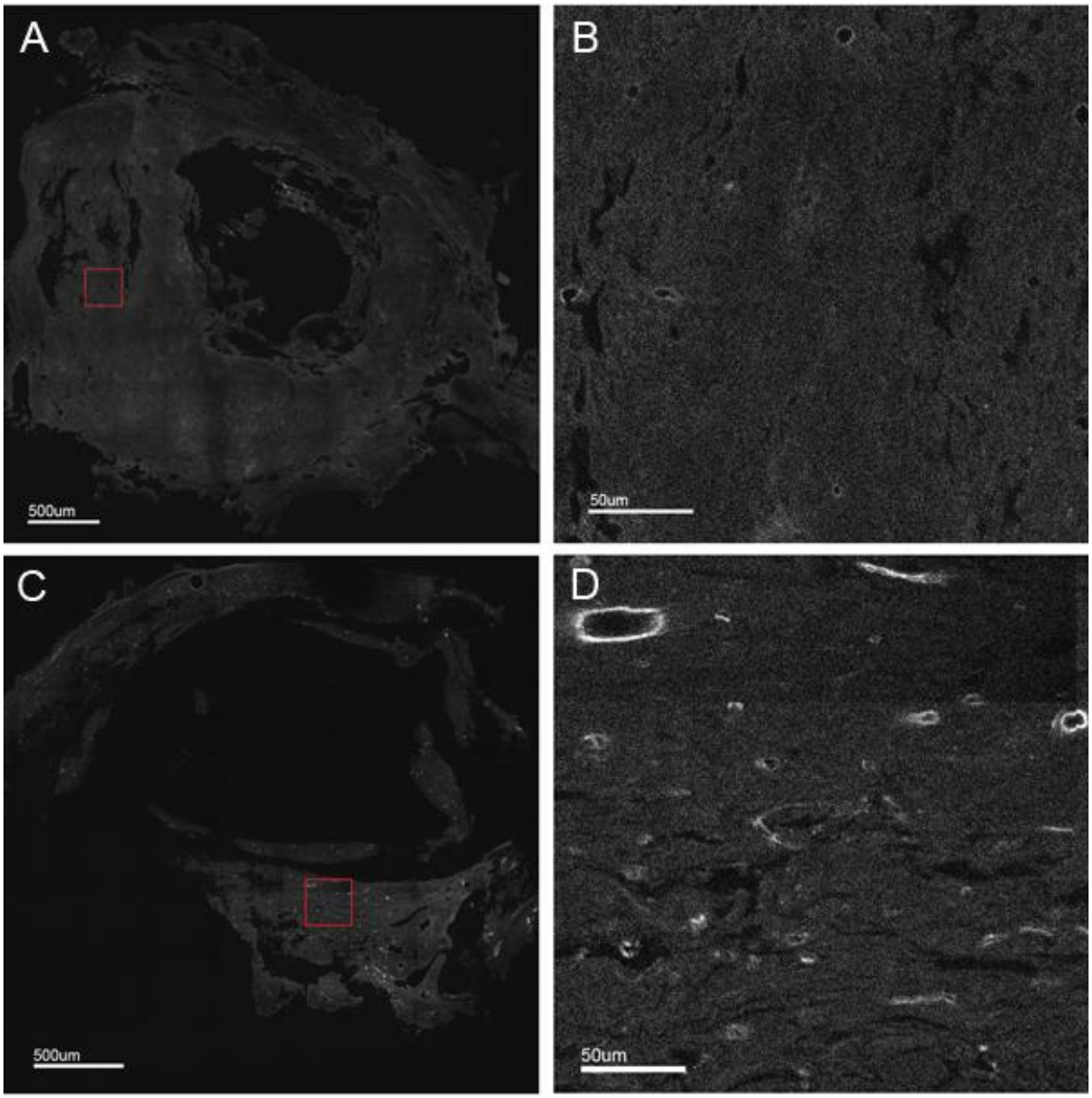
A
500um
B
50um
C
500um
D
50um

Figure 4.7 Two Month Scaffolds.(A) Confocal image of a 100mg/mL implant from a two month animal. A cystic cavity can be seen at the center of the tissue. (B) A close up view of vessels outlined in panel A. (C) Confocal image of a 150mg/mL implant from a two month animal. A very large cystic cavity has formed, leaving very little intact tissue. (D) Close up of the vessels outlined in panel C.

Discussion

The Angiogenic Response

Having a functioning blood supply is essential to the survival of an organism. It provides the essential nutrients needed to keep the cells of the body healthy and functioning. The ability of blood vessels to infiltrate damaged tissue is essential in creating and maintaining new tissue. In the case of spinal cord injury, harnessing the power of the angiogenic response may be the key to promoting regeneration and ultimately meaningful recovery.

Some experiments have shown the ability to stimulate angiogenesis using implants along with other factors included. One study showed that a bridge composed of micoporous hydrogel along with a neural progenitor/ endothelial cell culture could stimulate an angiogenic response (Rauch, M.F., 2009). Other approaches include collagen linked scaffolds with various cell types placed inside to stimulate a reparative response (Ramatullah,H.C., 2011). However, most bridging studies do not focus primarily on the angiogenic response, and if they do, there are often many variables which may be influencing this response. Our study focuses on one bridge with no other factors involved, to find out if altering the physical properties of the scaffold alters the angiogenic response.

We have seen that our electrospun scaffolds have the ability to induce an angiogenic response in the injured spinal cord. It has also been shown that by manipulating the fiber diameters in our scaffolds by altering the concentration, we can

manipulate the number of newly forming blood vessels. The ability of the scaffold to elicit and angiogenic response is a fascinating feature of our experimental model. The infiltrating blood vessels create an environment that is more suitable for regeneration to occur, but how is this process taking place? One theory is that as the scaffold is placed into the transected spinal cord, the severed vessels begin to infiltrate in a pattern that mirrors the original vascular architecture. The tightly grouped capillaries of the gray matter would be reflected within the scaffold. We would expect to see more vessels in the center of the scaffold than in the peripheral areas. We would also expect there to be larger caliber vessels seen in the outer areas, which would reflect the reestablishment of the arterial blood supply.

From a biological standpoint, the reason angiogenesis occurs is to fulfill the basic needs of the cellular environment. This includes supplying oxygen and carrying away carbon dioxide and other metabolic wastes from tissue, as well as supplying other essential nutrients. During the healing process, one of the first cellular responses is the establishment of new blood supply. This same reparative response is taking place at the site of injury in our animal model, and we believe that the scaffolds are providing a guide for regenerating blood vessels. One of the driving forces of the angiogenic response is lack of oxygen. In a hypoxic environment, cells begin to release VEGF which induces existing vessels to sprout and extend toward the area that requires oxygen. The scaffold is an area that is devoid of any blood supply initially, so we believe that any cells which migrate into the scaffold would begin to release VEGF in an attempt to recruit the necessary blood supply.

One possible explanation for the larger number of vessels in the 100mg/mL vs the 150mg/mL groups is a matter of space. The 100mg/mL scaffold has a tighter grouping of monofilaments than the 150mg/mL scaffold, along with fewer cavities. This provides a greater surface area for cellular infiltration in the 100mg/mL scaffold. Due to the greater number of cells entering the 100mg/mL scaffold, there would be a larger volume of VEGF being released which would stimulate a more robust angiogenic response. In the 150mg/mL scaffold, no cellular infiltration or blood vessel growth can take place in areas that have no monofilaments, making the number of cells entering the 150mg/mL scaffold fewer than the 100mg/mL scaffold. This would result in less VEGF being released from infiltrating cells, and therefore less angiogenesis would occur.

Histology

As we examine the histology, it appears that animals receiving the 100mg/mL implants show a greater angiogenic response than those that received the 150mg/mL implant. In both cases the number of vessels found in the center of the graft area began to increase over time, peak and then decrease at the latest time point. One explanation for this is the fact that at 1 week, cells have just begun to make their way into the scaffold, and the amount of VEGF being released is not as high as at the later time points. At one month the scaffolds are filled with cells, all releasing VEGF and in turn calling for a larger amount of vessels to meet their demands. At 2 months we see a decrease in the number of vessels. This may have to do with the natural pruning process that occurs after the initial angiogenic response; the vessels that are redundant or not required are removed. Another reason for the deceased number at 2 months is

the development of the cavities at the center of the lesion area, and there is simply less area for vessels to occupy.

At all three time points, the 100 groups displayed a greater number of vessels than the 150 groups overall. It was also observed that the increased vascularity produced by the 100mg/mL implants facilitated tissue sparing to a greater degree than the 150. Both animals in the 2 month groups developed cystic cavities, but the cavity formed in the 100 animal was significantly smaller in size. The cavity formed in the 2 month 150 animal was very large, leaving very little intact tissue. These initial findings suggest that our hypothesis regarding fiber diameter is correct. Smaller diameter fibers appear to increase angiogenesis, and that increased vascularity promotes greater tissue regeneration.

Methodology

Having compared the differences in histological outcomes using the two separate scaffolds at three distinct time points, we can see that there is a difference in angiogenic response produced by the two separate concentrations. There are some factors involved with the method of implantation that could be improved. The implantation surgery consisted of a thoracic laminectomy, followed by a complete spinal cord transection with a pair of microscissors. The implants were then inserted into the lesion area, and the surgical site was then closed. In the future, it may be beneficial to ensure the implant is secure in the lesion site and cannot be displaced when the animal moves about. This may help to increase the regenerative process by keeping the implant in the same orientation throughout the healing process, preventing possible damage to the

newly formed vasculature. While technically challenging, one approach to solving this problem would be to surgically deflect the dura before making the transection, and then sealing the dura after the implant is inserted. This could be done with suture or some type of surgical glue.

Limitations

There are many limitations associated with a study of this kind. The injury itself is quite traumatic, and the recovery of the animals post surgery can be difficult. The morbidity rate in this type of study is high, and losing animals due to complications limits the scope of the study. It is also difficult to ensure each animal has experienced the same amount of trauma; in some animals there is a full transection, while other animals appear to have a small amount of undamaged tissue at the ventral aspect of the cord. This inconsistency could produce varying degrees of difference in the final histological outcome.

Another factor that limited this particular study was the surgeons performing the surgeries. Two separate surgeons performed the surgeries in this study. Both had to be trained in the surgical technique, and the initial surgeries were of lesser quality than those performed later in the study. It is essential that in the future, the surgeon should be well trained in the proper surgical technique and its associated complications.

Limitations associated with the electrospinning process and the consistency of implant production need to be addressed as well. In some cases, the ability to electrospin effectively can be affected by factors such as the temperature or humidity in the spinning area. The overall diameter of the implant was also inconsistent in this

study; in some cases the implant itself was smaller than the diameter of the spinal cord. This limits the regenerative potential by not providing the proper structural support and guidance offered by the scaffold to the entire affected area.

Finally, the number of animals used in this particular study was low. Initial observations suggest that the 100mg/mL implant induces greater angiogenesis than the 150mg/ml implant. In order confirm that this is consistently the case, more animals need to be studied. This would include using the highest quality bridges, along with proper and consistent surgical technique.

Future Experiments

Directions for the future of the electrospun scaffolds will incorporate other factors within the implant itself to further aid in tissue sparing, regeneration, and functional recovery. It is thought that by employing a combinatorial strategy, we can increase the overall recovery of the animals. Factors that would be added to increase the overall effectiveness of the implant include both chemical and cellular cellular elements.

It has been shown in a previous study in our lab that the molecular fusogen, Polyethylene Glycol (PEG), increases the functional recovery of animals when used in conjunction with the implants (Dalton, 2011). It is believed that the use of PEG seals damaged axonal membranes, preventing neuronal cell death and promoting the sprouting of new axons. Another study conducted in our lab has shown that the incorporation of Schwann cells into the scaffold also increases functional recovery (Kanaan, 2012). It is thought that the Schwann cells are providing the necessary myelination of newly formed axons, allowing for proper signal transduction.

Future studies to promote regeneration following SCI will incorporate many factors. Using the proper fiber diameter to induce the greatest angiogenic response, coupled with the PEG and cell seeding could prove to be a very effective treatment strategy. This type of implant, coupled with proper and consistent surgical technique, has the potential to produce outstanding regenerative results and meaningful functional recovery. Using these types of strategies in the future has the potential to offer hope to the millions of people suffering from spinal cord injury.

List of References

Aird, W. C. (2012). Endothelial cell heterogeneity. *Cold Spring Harbor Perspectives in Medicine, 2*(1), a006429.

Arese, M., Serini, G., & Bussolino, F. (2011). Nervous vascular parallels: Axon guidance and beyond. *The International Journal of Developmental Biology, 55*(4-5), 439-445.

Bautch, V. L., & James, J. M. (2009). Neurovascular development: The beginning of a beautiful friendship. *Cell Adhesion & Migration, 3*(2), 199-204.

Benarroch, E. E. (2012). Blood-brain barrier: Recent developments and clinical correlations. *Neurology, 78*(16), 1268-1276.

Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C., & Vescovi, A. L. (1999). Turning brain into blood: A hematopoietic fate adopted by adult neural stem cells in vivo. *Science (New York, N.Y.), 283*(5401), 534-537.

Bunge, M. B. (2008). Novel combination strategies to repair the injured mammalian spinal cord. *The Journal of Spinal Cord Medicine, 31*(3), 262-269.

Cassell, O. C., Hofer, S. O., Morrison, W. A., & Knight, K. R. (2002). Vascularisation of tissue-engineered grafts: The regulation of angiogenesis in reconstructive surgery and in disease states. *British Journal of Plastic Surgery, 55*(8), 603-610.

Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., et al. (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: A mechanism of O2 sensing. *The Journal of Biological Chemistry, 275*(33), 25130-25138.

Chow, W. N., Simpson, D. G., Bigbee, J. W., & Colello, R. J. (2008). Evaluating neuronal and glial growth on electrospun polarized matrices: bridging the gap in percussive spinal cord injuries. *Neuron*, *3*(2), 119-126.

Cholas, R. H., Hsu, H. P., & Spector, M. (2012). The reparative response to cross-linked collagen-based scaffolds in a rat spinal cord gap model. *Biomaterials, 33*(7), 2050-2059.

D'Aversa, T. G., Eugenin, E. A., Lopez, L., & Berman, J. W. (2012). Myelin basic protein induces inflammatory mediators from primary human endothelial cells and blood-

brain-barrier disruption: Implications for the pathogenesis of multiple sclerosis. *Neuropathology and Applied Neurobiology,*

Dalton, D. (2011) Assessing the role of Poly Ethylene Glycol (PEG) in improving functional recovery Following spinal cord injury. *Master's Thesis.*

Emery, E., Aldana, P., Bunge, M. B., Puckett, W., Srinivasan, A., Keane, R. W., et al. (1998). Apoptosis after traumatic human spinal cord injury. *Journal of Neurosurgery, 89*(6), 911-920.

Fawcett, J. W. (2006). Overcoming inhibition in the damaged spinal cord. *Journal of Neurotrauma, 23*(3-4), 371-383.

Flaherty, J. T., Pierce, J. E., Ferrans, V. J., Patel, D. J., Tucker, W. K., & Fry, D. L. (1972). Endothelial nuclear patterns in the canine arterial tree with particular reference to hemodynamic events. *Circulation Research, 30*(1), 23-33.

Hagg, T., & Oudega, M. (2006). Degenerative and spontaneous regenerative processes after spinal cord injury. *Journal of Neurotrauma, 23*(3-4), 264-280.

Kannan, A. (2012). Combining electrospun polydioxanone scaffolds, Schwann cells, and Matrigel to improve functional recovery after a complete spinal cord transection in rats. *Master's Thesis.*

Lin, C.S. (2007). Spinal cord injury: exploring the histology of electrospun implants in vivo. *Master's Thesis.*

Mackenzie, F., & Ruhrberg, C. (2012). Diverse roles for VEGF-A in the nervous system. *Development (Cambridge, England), 139*(8), 1371-1380.

Martirosyan, N. L., Feuerstein, J. S., Theodore, N., Cavalcanti, D. D., Spetzler, R. F., & Preul, M. C. (2011). Blood supply and vascular reactivity of the spinal cord under normal and pathological conditions. *Journal of Neurosurgery.Spine, 15*(3), 238-251.

Mautes, A. E., Weinzierl, M. R., Donovan, F., & Noble, L. J. (2000). Vascular events after spinal cord injury: Contribution to secondary pathogenesis. *Physical Therapy, 80*(7), 673-687.

Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G. D., & Crain, B. (2003). Transplanted bone marrow generates new neurons in human brains. *Proceedings of the National Academy of Sciences of the United States of America, 100*(3), 1364-1369.

Norenberg, M. D., Smith, J., & Marcillo, A. (2004). The pathology of human spinal cord injury: Defining the problems. *Journal of Neurotrauma, 21*(4), 429-440.

Pepper, M. S., Mandriota, S. J., Vassalli, J. D., Orci, L., & Montesano, R. (1996). Angiogenesis-regulating cytokines: Activities and interactions. *Current Topics in Microbiology and Immunology, 213 (Pt 2)*(Pt 2), 31-67.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature, 386*(6626), 671-674.

Rahmatullah, H.C., Hsu, H.P., Spector, M. (2012) The reparative response to cross-linked collagen-based scaffolds in a rat spinal cord gap model. *Biomaterials,33,* 2050-2059.

Rauch, M.F., et al. (2009). Engineering angiogenesis following spinal cord injury. *The European Journal of Neuroscience, 29*(1), 132-145.

Rooney, G. E., Endo, T., Ameenuddin, S., Chen, B., Vaishya, S., Gross, L., et al. (2009). Importance of the vasculature in cyst formation after spinal cord injury. *Journal of Neurosurgery.Spine, 11*(4), 432-437.

Schnell, L., Fearn, S., Klassen, H., Schwab, M. E., & Perry, V. H. (1999). Acute inflammatory responses to mechanical lesions in the CNS: Differences between brain and spinal cord. *The European Journal of Neuroscience, 11*(10), 3648-3658.

Sofroniew, M. V. (2005). Reactive astrocytes in neural repair and protection. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry, 11*(5), 400-407.

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in Neurosciences, 32*(12), 638-647.

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica, 119*(1), 7-35.

Whetstone, W. D., Hsu, J. Y., Eisenberg, M., Werb, Z., & Noble-Haeusslein, L. J. (2003). Blood-spinal cord barrier after spinal cord injury: Relation to revascularization and wound healing. *Journal of Neuroscience Research, 74*(2), 227-239.

White, R. E., & Jakeman, L. B. (2008). Don't fence me in: Harnessing the beneficial roles of astrocytes for spinal cord repair. *Restorative Neurology and Neuroscience, 26*(2-3), 197-214.

Vita

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