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Nithya Jesuraj
Washington University in St. Louis

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Shelly Sakiyama-Elbert, Chair
Dennis Barbour
Paul Bridgman
Donald Elbert
Philip Johnson
Kelly Monk

Evalulating Environmental Cues that Affect Schwann Cell Gene Expression to Enhance

Peripheral Nerve Regeneration

by

Nithya Jothi Jesuraj

A dissertation presented to the
Graduate School of Arts and Sciences
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ABSTRACT OF DISSERTATION

EVALUATING ENVIRONMENTAL CUES THAT AFFECT SCHWANN CELL
GENE EXPRESSION TO ENHANCE PERIPHERAL NERVE REGENERATION

By

Nithya Jothi Jesuraj

Doctor of Philosophy in Biomedical Engineering
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Chairperson: Shelly Sakiyama-Elbert, Ph.D.

The goal of this thesis was to evaluate the effects of different environmental cues on Schwann cell (SC) differentiation and phenotype maintenance to design better SC transplantation therapies for peripheral nerve repair. First, a set of markers, specific for motor or sensory-derived SCs were identified from the literature and gene chips. After 30 days, gene expression patterns of SCs, after expansion in culture, were dysregulated. Cues that have been hypothesized to re-differentiate the SCs *in vitro* are extracellular matrix (ECM) molecules, growth factors (GFs), and acetylcholine (Ach). To test the effects of ECM, SCs were transplanted into acellular nerve grafts (ANGs), which have an intact ECM, and were used to repair a 14 mm rat sciatic nerve injury. After 2 weeks, the RNA was analyzed for expression levels of GFs (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and glial cell derived neurotrophic factor (GDNF)) and also phenotypic markers. The phenotype-specific SCs expressed higher levels of NGF, BDNF, and GDNF compared to levels in the injuries repaired with an isograft. The expression patterns of the phenotypic markers were still disrupted at 2 weeks post transplant suggesting that other cues (GFs or Ach) are necessary to promote native marker expression. The addition of GFs NGF and GDNF to SCs in culture promoted increased mature marker expression (S100) over a period of 7 days. Evaluation of expression patterns showed sensory-derived SCs treated with NGF had increased expression of sensory markers and motor-derived SCs had no detectable expression of sensory markers. GDNF promoted the correct phenotypic marker expression in both sets of SCs treated. Finally, Ach was added to motor-derived SC cultures to determine if it had an effect on the phenotypic maintenance of motor-derived SCs. In addition, Ach receptors were blocked with gallamine to test the specificity of the Ach effect. Gene expression analysis showed that Ach promoted increased expression of motor markers in motor-derived SCs, and that gallamine blocked the effects of Ach. Overall, this work has shown that environmental cues, such as ECM, GFs, and Ach affect SC phenotype and differentiation.

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Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1   Overview

This work seeks to understand how a Schwann cell's (SC's) environmental cues (extracellular matrix (ECM), growth factors (GFs) and neurotransmitters) influence differentiation into mature SCs to guide and remyelinate regenerating axons after peripheral nerve injury. SCs are the support cells of the peripheral nervous system (PNS). In addition to myelinating and providing trophic support for axons, SCs are also involved in aiding the regeneration of axons post-injury. SCs secrete ECM molecules, as well as GFs, to guide regenerating axons to their target end-organs. In an uninjured nerve, Hoke *et al.* have shown that SCs derived from motor or sensory nerve sources exhibit different phenotypes, which may influence the pathway a regenerating motor or sensory axon chooses. Therefore, transplanting SCs derived from different sources may provide the trophic support necessary to influence the pathway a regenerating motor or sensory axon chooses. However, prior to transplantation of SCs at the injury site, SCs must be expanded *in vitro* to obtain a sufficient number of cells. To study SC gene expression patterns during expansion, SCs derived from the motor and sensory branches of the rat femoral nerve were cultured *in vitro* and expression pattern changes were compared to fresh tissue. Additionally, SC gene expression patterns were studied in the presence of a variety of environmental cues including ECM (basal lamina microstructure and laminin), GFs shown to promote nerve regeneration, and acetylcholine (Ach), a small molecule neurotransmitter that may be present in the SC environment.

The first study was done to establish a baseline gene expression pattern for SCs

derived from different nerve sources. SCs were harvested and expanded *in vitro* on poly-L-lysine plates from both the motor and sensory branches of the rat femoral nerve. To determine markers that are differentially upregulated in either motor-derived SCs or sensory-derived SCs, fresh RNA was extracted from nerve tissue and analyzed using Affymetrix gene chips and qRT-PCR. From this data, 4 markers for each phenotype (2 from literature, 2 from gene chip analysis) were selected for monitoring the gene expression patterns of SCs. RNA was extracted from SCs in culture at days 1, 3, 7, 14 and 30 and gene expression was compared to expression on day 0 (fresh nerve tissue). In this study it was shown that SCs derived from motor and sensory nerve were phenotypically different and that gene expression patterns were dysregulated as SCs were expanded *in vitro.* This loss of phenotypic identity may limit the influence of SC phenotypes on regeneration. Investigation into which aspects of the SCs environment may affect redifferentiation of SCs into their original phenotype, which is important to improve SC transplantation therapies.

Using the gene expression results, the effects of ECM on SC differentiation was studied by transplanting SCs into ANGs. To evaluate these effects, SCs were expanded in culture and transplanted into acellular nerve grafts (ANGs), which were used to treat a 14 mm rat sciatic nerve injury. Transplanting SCs into ANGs to treat a nerve injury will help in understanding of whether the ECM in a SC's environment may promote differentiation of SCs back into their original phenotype. The ANGs were donor rat sciatic nerves that were harvested and processed in University of Wisconsin (UW) solution for 7 weeks, which greatly reduces the immunological response to cellular components of the graft (Southard and Belzer 1995). Additionally, the resulting cold-preserved ANG (CP-ANG) was stripped of cells but has an intact endoneurial microstructure of ECM. SCs were injected under the epineurium of the CP-ANG and then the CP-ANGs were used to treat the nerve injury.

Two weeks post-transplantation the nerves were explanted and RNA was extracted and analyzed for phenotypic marker and growth factor gene expression. The addition of SCs promoted increased expression of NGF, BDNF, and GDNF (shown to aid in peripheral nerve regeneration), but failed to promote the original phenotypic marker expression in ANGs with motor or sensory-derived SCs. It can be seen from this study that the intact ECM of a CP-ANG does not promote differentiation of SCs into their native phenotype, which suggests that additional cues are necessary to promote differentiation of SCs.

The final study was done to evaluate the effects of GFs and small molecules that may affect the differentiation of SCs. In the SC environment, GFs, such as NGF or GDNF are present to support and promote neuronal survival. During injury, SCs de-differentiate to proliferate and increase secretion of GFs, including NGF and GDNF, into the environment. This stage promotes and guides axonal regeneration from the proximal to the distal nerve stump. As axons regenerate, autocrine signaling (possibly due to GFs) in SCs allow the SCs to redifferentiate into their native phenotypes. Because SCs express both the $p75^{NTR}$ (NGF) and GFRα-1 receptors (GDNF), it is possible that NGF and GDNF may promote the signaling necessary for differentiation of SCs back into their native phenotype. In addition, small molecules, such as acetylcholine (Ach), are also present in the environment of motor-derived SCs near the neuromuscular junction (NMJ). Although Ach may not promote the differentiation of SCs, it may help reinforce the phenotype of motor-derived SCs and promote increased expression of phenotype-specific markers in motor-derived SCs. Dosage studies were performed to determine optimal doses of NGF, GDNF, and Ach that may promote differentiation of SCs derived from mature motor or sensory nerves. The addition of NGF and GDNF to the media promoted the differentiation of both sensory and motor-

derived SCs back into their original phenotypes. Ach addition to motor-derived SCs

promoted differentiation and increased motor-specific marker expression.

This introduction will take a deeper look at the characteristics and mechanisms

behind peripheral nerve injury, as well as the importance of SCs and environmental cues to

promote peripheral nerve regeneration. Specifically, understanding certain cues that

influence SC differentiation may help in designing better SC transplantation therapies for

peripheral nerve injury. Additionally, current and past treatments for peripheral nerve injury

including biological grafts, nerve guidance conduits, and SC transplantation will be discussed

to establish the state of the field.

1.2   Peripheral Nerve Injury and Regeneration

The nervous system consists of two branches: central nervous system (CNS) and

peripheral nervous system (PNS). The CNS is the largest part of the nervous system that

includes the brain and spinal cord. In conjunction with the PNS, it serves as the primary

controller of behavior and is protected by bone. The PNS extends outside of the CNS and

serves to control the movement of limbs. Unlike the CNS, the PNS is not protected by

bone and is more prone to exposure to toxins and mechanical injuries (Bunge 1993;

Reynolds and Woolf 1993). PNS injuries are commonly due to accidents that cause

stretching, laceration, or compression of the nerves and represent a large portion of nerve

repair procedures performed annually (Beazley, Milek et al. 1984; Dellon and Mackinnon

1988; Kouyoumdjian 2006). Damage to the PNS can result in impaired motor and/or

sensory nerve function at denervated end-organs. The PNS is capable of limited

regeneration; however, treating axonal injury still remains a clinical challenge (Burnett and

Zager 2004). This section serves to describe peripheral nerve injury and as the issues that surround nerve regeneration.

1.2.1 Characteristics of Injury and Regeneration

Peripheral nerves mainly control movements and sensory perception. They consist of nerve fascicles that are a mixture of motor and sensory axons, some of which are myelinated by SCs. Each nerve fascicle consists of different layers: the inner endoneurium, the perineurium that surrounds each fascicle, and the epineurium that bundles the nerve fascicles and forms a nerve. Injuries to the PNS can involve damage to one or more of these layers, with more serious damage resulting in a crush or complete transection of the nerve (Burnett and Zager 2004).

Following injury, a phenomenon called Wallerian degeneration occurs at the injury site due to the separation of the axon from the cell body (Waller 1850). However, the neurons can survive injury and cell death by upregulating the growth factors responsible for axonal growth and survival. In addition, the distal stump also undergoes many significant changes to prepare for regenerating axons from the proximal stump. During the first week after injury, glial cells (i.e. SCs) and macrophages infiltrate the distal stump to phagocytose the myelin and axonal debris in the area that may be inhibitory to axonal regeneration (Waller 1850; Bruck 1997). After clearance of the debris, SCs start proliferating in response to signaling from the axonal membrane, the myelin debris, and possibly from the macrophages at the injury site (Williams and Hall 1971; Baichwal, Bigbee et al. 1988). These proliferating SCs align themselves in the remaining basal lamina and endoneurial tubes in the distal stump to form bands of Bungner, which are tubes that act as a natural support for the regenerating axons and guide them back to the correct target end-organ (Burnett and Zager 2004). Following nerve transection, axons begin sprouting from the proximal nerve and

5

grow towards the distal stump by following the aligned SCs in the endoneurial tubes (Haftek and Thomas 1968). These axon sprouts are guided by cell adhesion and ECM molecules produced by SCs. SCs primarily produce replacement basal lamina consisting of laminin, type IV collagen, and fibronectin (Rogers, Letourneau et al. 1983; Fawcett and Keynes 1990). Additional trophic support for the regenerating axons is provided by the neurotrophic factors secreted by SCs and possibly by the target end-organ (e.g. muscle or skin). This additional support promotes neurite survival, migration, and synapse formation at the end-organ (Reichardt and Tomaselli 1991). After migrating through the endoneurial tubes, the axons are myelinated by SCs, and reestablish connections with target end-organs. Correct reinnervation of the end-organ signals that the axons are fully matured.

1.2.2 Challenges in Peripheral Nerve Regeneration

Although the PNS has the capacity for regeneration, axonal regeneration often does not occur successfully in larger nerve defects. The clinical treatment used in these cases does not necessarily restore complete nerve function. The degree of regeneration, motor or sensory, depends greatly on the treatment used (Rogers, Letourneau et al. 1983; Fawcett and Keynes 1990). Also, multiple sprouts that extend from the proximal to the distal stump may encounter a number of difficulties. The regenerating axons may not reinnervate the correct end-organ target (Wigston and Donahue 1988; Kingham and Terenghi 2006). When improper connections are established these branches are eliminated (Aitken 1949). Even when the axons successfully reinnervate an end-organ, such as muscle, they tend to form new connections that were not previously established. Any muscle endplate can be reinnervated by any motor nerve fiber, which can result in improper innervations and thus loss of coordination or complex improper functioning of other associated muscles due to

insufficient muscle contraction (Kingham and Terenghi 2006). Additionally, the slow and

fast twitch muscle fibers are not preserved in their original form. Even with appropriate

muscle reinnervation, full functional recovery is difficult due improper sensory reinnervation

of the muscle (Burnett and Zager 2004).

Many hypotheses on motor versus sensory nerve regeneration and axonal guidance

exist. One hypothesis is that the target end-organ provides diffusible cues or necessary

trophic support to guide axons to reinnervate the correct end-organ (Madison, Robinson et

al. 2007). Regenerating axons follow a chemotropism in which they grow towards the distal

nerve stump rather than other tissue (Fawcett and Keynes 1990). Madison and colleagues

have extended this idea of chemotropism to guide the axons down the correct end-organ

pathways by demonstrating that motor axons rely on trophic support from the end-organ to

properly find and reinnervate their targets. These findings were based on a series of

experiments in the rat femoral nerve by modulating the distance axons travel to their end-

organ targets after injury (Madison, Robinson et al. 2007; Uschold, Robinson et al. 2007).

This theory is also supported by the observation that both motor and sensory axons contain

different cell surface receptors for guidance cues (Boyd and Gordon 2003). Alternatively, it

has been suggested that SCs present in the endoneurial tubes provide axonal guidance cues

to promote the reinnervation of the correct end-organ (Politis 1985; Brushart 1988; Wigston

and Donahue 1988). Alternatively, in nerve crush injuries the endoneurial tubes are

preserved and axons are able to regenerate in their parent endoneurial tubes and reestablish

optimal levels of functional recovery after injury (Haftek and Thomas 1968). From these

results, it may be inferred that the disruption of endoneurial tubes leads to inappropriate

reinnervation of the end-organ. Also, additional research in preferential motor reinnervation

(PMR) in the rat femoral nerve model led to the hypothesis that endoneurial tubes or SCs

within the tubes may guide axons down the correct pathways (Brushart 1988). Overall, axonal guidance to the target end-organ depends on a variety of factors: endoneurial tubes, SCs, diffusible cues, and the end-organ. Therefore, to design adequate therapies to promote correct axonal regeneration all of these factors need to be considered.

1.3 Current Treatment Options

As previously mentioned, a complete nerve transection in the peripheral nerve leading to a disconnection between the proximal and distal nerve stumps may result in complete loss of motor or sensory function due to loss of nerve connection to the end-organ. In humans, the average rate of axonal regeneration is 1 mm/day, which makes this a slow process for injuries that are far from the injury site (Evans 2001; Burnett and Zager 2004). However, when the gap between the two nerve ends is large, axonal growth is limited and surgical intervention is often required (Lundborg 2000). To promote nerve regeneration, many nerve repair strategies have been used to bridge the gap and allow the migration of glial cells and surviving axons to grow from the proximal stump to the distal stump. The PNS is capable of regeneration when the two nerve ends can be joined back together by direct anastomosis. This allows the distal stump to provide the necessary support via SCs and neurotrophic factors to guide regenerating axons (Burnett and Zager 2004). However, in larger defects, the suture would introduce unnecessary tension that may prevent normal regeneration (de Medinaceli, Wyatt et al. 1983). Therefore, a bridge or scaffold must be used to reconnect the proximal and distal stumps. This section describes materials and strategies that are used to bridge the nerve injury gap.

1.3.1 Nerve Guidance Conduits (NGCs)

For many years, NGCs have been extensively studied as a potential "off the shelf" alternative to nerve grafts for the treatment of nerve injury. NGCs typically consist of a hollow conduit that can be filled with an ECM scaffold containing trophic factors and other cues for regenerative support for axons. A great deal of research has been conducted on the construction of biodegradable conduits, but they may present biocompatibility issues. One main advantage of using conduits is that they isolate the environments where the regeneration is occurring and allow for the controlled release of cues to examine their effects on the regenerating axons.

The first use of conduits to bridge a nerve defect was done by Lundborg. However, his initial motivation was to study peripheral nerve regeneration and not to bridge a nerve gap defect. Initially, they used a pseudosynovial neural sheath to repair a sciatic nerve injury but later switched to silicone conduits to repair the nerve defect (Lundborg and Hansson 1979; Lundborg and Hansson 1980). They discovered that both materials led to a convenient method to contain trophic factors that have been implicated in supporting nerve regeneration and to study the mechanisms of nerve regeneration through the conduits. These experiments also showed that a cellular scaffold of natural proteins and glial cell migration followed axonal sprouting into an empty tube, suggesting that even in the absence of any regenerative cues, the peripheral nerves are capable of some regeneration (Lundborg and Hansson 1979; Lundborg and Hansson 1980; Lundborg, Dahlin et al. 1982; Lundborg, Dahlin et al. 1982).

Silicone conduits have been used to treat small nerve defects in humans (Lundborg, Dahlin et al. 1991; Lundborg, Rosen et al. 1997; Lundborg, Rosen et al. 2004). Although silicone is a biocompatible material and is mechanistically stable, there have been reports of

associated morbidities. Silicone conduits have been reported to cause nerve compression and irritation at the implantation site, requiring immediate removal (Dellon and Mackinnon 1988; Merle, Dellon et al. 1989; Dellon 1994; Dahlin and Lundborg 1999). The long terms effects of silicone present in the surrounding nerve may have caused the condition and thus other materials have been researched as replacements.

Numerous groups have constructed and studied the properties of various conduit materials, such as poly-L-glycolic acid (Mackinnon and Dellon 1990; Hadlock, Elisseeff et al. 1998), poly (lactic-co-glycolic acid) copolymer (Hadlock, Elisseeff et al. 1998), poly(L-lactide-co-6-caprolactone) (Nicoli Aldini, Perego et al. 1996), and vinylidenefluoride-trifluoroethylene copolymer (Fine, Valentini et al. 1991). These materials were selected mainly because of their range of degradation, mechanical stability, and piezoelectric properties (vinylidenefluoride-trifluoroethylene copolymer only) that may benefit nerve regeneration. Alternatively, naturally derived materials can also be used as biodegradable conduits. Advantages of these materials may include increased permeability of oxygen and nutrients to the injury site and increased biocompatibility over synthetic materials. The repair of peripheral nerves with a crosslinked collagen conduit has shown promise in terms of its ability to promote nerve regeneration and still provide structural support for the regenerative axons. In both rodent and primate short nerve defect models, these collagen conduits showed increased regeneration in terms of functional recovery compared to direct suturing techniques or autologous graft repair (Archibald, Krarup et al. 1991). Alternatively, other ECM molecules, fibronectin and fibrin, have been oriented in mats to produce conduits to promote nerve regeneration (Whitworth, Brown et al. 1995; Kalbermatten, Pettersson et al. 2009). More recently, engineered bi-layer nanofiber conduits have been

shown to have mechanical stability to promote regenerating and therapeutic effects similar to autografts (Zhu, Wang et al. 2011).

Overall, NGCs support peripheral nerve regeneration and provide valuable insight into the regeneration process. It has been shown that synthetic and naturally-derived NGC materials can promote peripheral nerve regeneration in small nerve defects (<10 mm) and in some cases larger nerve defects (>10 cm). However, NGCs may not provide enough cues to guide axons and obtain complete functional recovery. These conduits can be improved by filling the lumen with ECM scaffolds containing neurotrophic factors and the support cells native to the injury site, such as SCs. However, the materials chosen for conduits and luminal fillers must be biocompatible, which is a challenge in the field. In the Sakiyama-Elbert lab, it has been shown that the inclusion of fibrin-based affinity drug delivery system (Sakiyama-Elbert and Hubbell 2000; Sakiyama-Elbert and Hubbell 2000) into the lumen of silicone conduits can promote the regeneration of the rat sciatic nerve in a 14 mm nerve gap defect (Lee, Yu et al. 2003; Wood, Moore et al. 2009; Wood, Macewan et al. 2010). But as mentioned earlier, silicone conduits can cause chronic nerve compression and irritation at the injury site, and require additional surgery to remove the conduits. Alternative approaches to bridge nerve gap defects that have been researched include biological grafts, which contain biological material native to the environment.

1.3.2 Biological Grafts

The first surgeries using biological grafts to bridge critical nerve defects were performed in the 1960's (Millesi 1973). Despite significant advances in nerve reconstruction since that time, the autologous nerve graft remains the clinical gold standard for critical, long, peripheral nerve defect repair. The autograft provides the necessary scaffold and

11

trophic support, basal lamina, endoneurial tubes, and SCs to guide the regenerating axons from the proximal stump to the distal stump (Mackinnon 1989; Belkas, Shoichet et al. 2004)). Donor nerves are commonly taken from the sural (cutaneous) nerve or other sensory nerves (Meek and Coert 2002). Unfortunately, this method provides many limitations, such as donor site morbidity, lack of sufficient donor tissue, or size mismatches at the injury site (Schmidt and Leach 2003; Burnett and Zager 2004). Additionally, functional recovery with nerve autografts is not ideal. Less than 25% of the patients that received treatment regained full motor function and only 1-3% of the patients recovered normal sensation after 5 years (Dellon and Mackinnon 1988). Even with appropriate matching of fascicles during surgery, axonal guidance to the correct fascicles and reinnervation of the correct end-organ is not guaranteed (Gordon, Sulaiman et al. 2003). Although autografts need improvement, the suboptimal results of autografts may be due to the source of the graft. It has been shown that motor nerve repairs performed with a motor graft was superior to a sensory graft in terms of increased nerve density, percent nerve and total fiber number (Brenner, Hess et al. 2006). Similarly, a mixed nerve defect (i.e. containing both motor and sensory axons) when repaired with a mixed or motor graft resulted in better regeneration than when the defect was repaired with a sensory graft (Nichols, Brenner et al. 2004). Although better regeneration is seen with a motor graft, obtaining donor motor nerves are impossible due to their essential function. Therefore, alternative grafts have been considered that may promote increased nerve regeneration.

Nerve allografts have been considered as alternatives to nerve autografts. Allografts are taken from a member of the same species, but require immunosuppression to avoid rejection by the patient. Many studies have been conducted by the Mackinnon lab to studies the use of allografts as alternatives to autografts. They have tested antibodies to induce

antigen-specific tolerance, which allows the allograft to perform as well as isografts in nerve histomorphometry measures in mice and rats (Nakao, MacKinnon et al. 1995; Nakao, Mackinnon et al. 1995). Antibody studies using anti-CD40 to block the CD40/CD40 ligand interaction in mouse and primate models showed similar nerve regeneration results to the autografts (Brenner, Jensen et al. 2004; Jensen, Tung et al. 2004). However, when the treatments were stopped, the allografts were rejected by the body (Brenner, Jensen et al. 2004; Jensen, Tung et al. 2004). To avoid chronic immune suppression, commercially available acellular allografts (from Axogen, Inc) or processed allografts can be used to bridge nerve defects. Different processing techniques (freeze thaw cycles and cold-preservation (Gulati and Cole 1994), detergents (Hudson, Liu et al. 2004; Hudson, Zawko et al. 2004)) strip the graft of cells thus reducing the immunological response to cellular components. Compared with nerve conduits, processed allografts support superior regeneration, likely due to the intact endoneurial microstructure of ECM proteins (Whitlock, Tuffaha et al. 2009; Johnson, Newton et al. 2011). The Mackinnon lab has shown that decellularized allografts do not perform as well as the isografts as assessed by histology and electrophysiology in bridging a gap larger than 14 mm (Whitlock, Tuffaha et al. 2009). The presence of SCs in the isograft is hypothesized to increase nerve regeneration compared to the decellularized graft, which lacks SCs. Other researchers have also concluded that the lack of cellular support in acellular grafts results in the inferior nerve regeneration to autografts (Gulati 1988).

In addition to autografts and allografts, other biological tissues with matching dimensions and increased availability have been assessed as grafts. Vein or artery tissue has been used as an alternative to nerve autografts due to its tubular structure similar to conduits (Foidart-Dessalle, Dubuisson et al. 1997). However, vein and artery tissue may provide

obstacles for regeneration because of the mechanical properties. The thin walls of the tissue may collapse leading to excess pressure on the regenerating axons. Tissue mismatching may also lead to scarring. Muscle tissues offer a better alternative due to a basal lamina organization that mimics the endoneurial tube structure of peripheral nerves, and it contains collagen and laminin, which promote nerve outgrowth (Glasby, Gschmeissner et al. 1986; Norris, Glasby et al. 1988; Belkas, Shoichet et al. 2004). However, mechanical dissimilarities to nervous tissue and scarring due to size mismatch has been observed when muscle tissue has been used for nerve regeneration (Meek and Coert 2002).

It can be seen that using nerve autografts, nerve allografts, processed allografts, or other biological tissues may not provide the necessary support for functional recovery due to tissue size mismatches, donor site morbidity, lack of donor tissue, or lack of cellular support. The possible transplantation of SCs or addition of other trophic support to decellularized graft may promote increased nerve regeneration and provide a better alternative for peripheral nerve repair strategies.

1.4   Schwann Cells (SCs)

SCs are derived from the neural crest and differentiate with cues from the environment into two types of SCs: myelinating and nonmyelinating (Mirsky and Jessen 1996). Myelinating SCs associate with axons in a 1:1 ratio and aid in saltatory conduction. Alternatively, nonmyelinating SCs associate with several non-myelinated axons that conduct signals with wave-like impulses (Mirsky and Jessen 1996; Jessen and Mirsky 2005). In uninjured nerves, SCs secrete ECM molecules and growth factors (GFs) to promote neuronal survival and guide regenerating axons to their distal targets. In addition to supporting neurons and myelinating axons, SCs play an integral role in promoting nerve

regeneration after injury. Due to the loss in trophic support from the end-organ, SCs are necessary to promote axonal regeneration. In this section, the main roles of SCs in nerve regeneration will be discussed.

1.4.1   Role in Peripheral Nerve Regeneration

Wallerian degeneration follows axonal injury within 24-36 hours during which the axons disintegrate and axonal membranes break apart. The degeneration is followed by degradation of the myelin sheath and infiltration by macrophages in the distal stump. SCs and macrophages phagocytose and clear myelin debris (Waller 1850; Stoll, Griffin et al. 1989; Stoll, Trapp et al. 1989). During this phase, SCs along with fibroblasts upregulate their secretion of neurotrophic factors to provide a favorable environment for axonal growth and regeneration. Besides secreting growth factors, SCs also provide structural support to guide regeneration axons by proliferating and forming bands of Bungner within the basal lamina tubes guiding the regenerating axon from the proximal to the distal stump to reinnervate the target end-organ (Reynolds and Woolf 1993; Bunge 1994; Nagarajan, Le et al. 2002; Brenner, Lowe et al. 2005).

Studies in the past have also implied that SCs may have possible roles in promoting regeneration of axons down either a motor or sensory pathway. When given equal access to motor and sensory pathways, it has been shown that motor axon tends to regenerate down the motor pathway (PMR). In many studies, the Brushart and Madison labs have shown that regenerating motor axons preferentially regenerate down the quadriceps pathway even when deliberately trying to mismatch motor and sensory paths. It has been hypothesized that this regeneration phenomenon is influenced by trophic support from the end-organ (with muscle greatly outweighing skin in PMR) or SCs present within terminal nerve pathways (Brushart

1988; Brushart 1993; Madison, Archibald et al. 1996; Madison, Archibald et al. 1999; Madison, Robinson et al. 2007; Madison, Sofroniew et al. 2009). Although most of the research on nerve regeneration specificity has focused on PMR, Hoke *et al.* have shown that sensory axons may also regenerate with similar specificity down a sensory pathway. The specificity with which these axons regenerate may be due to phenotypic differences present within the regenerating pathway and SCs in the endoneurial tubes that are guiding the regenerating axons (Hoke, Redett et al. 2006). Although SCs de-differentiate, they may retain a "phenotypic memory" that allows them to re-differentiate into their original phenotype during regeneration, as evidenced by different expression profiles observed by motor and sensory SCs even after prolonged contact with axons of the opposite phenotype (Hoke, Redett et al. 2006).

1.4.2 Schwann Cell (SC) Transplantation

The transplantation of SCs into NGCs or biological grafts has been previously studied to understand the effects of SCs on peripheral nerve regeneration. As mentioned earlier, SCs provide trophic support and help to drive targeted regeneration and myelination of injured axons. SCs support nerve regeneration *in vivo* by producing basal lamina and neurotrophic factors and cell-cell adhesion molecules (Bunge, Bunge et al. 1986; Bunge 1994; Burnett and Zager 2004). After injury and denervation, SCs de-differentiate into a more immature state, and both types of SCs produce the same growth factors regardless of whether they had been associated with either myelinated or nonmyelinated axons (Mirsky and Jessen 1996; Jessen and Mirsky 2005). Because of this regenerative support from SCs, much research has been conducted to study the effects of transplanting SCs to promote nerve regeneration.

Schwann cells, when cultured *in vitro,* retain the ability to express myelin and neurotrophic factors and thus have been transplanted into the CNS or PNS to promote nerve regeneration. The addition of SCs to laminin-filled NGCs was found to increase the fasciculation of the regenerating nerve. Other researchers have shown that although SC-seeded NGCs lactic and glycolic acid (PLGA) foam conduits (Hadlock, Sundback et al. 2000) or poly(L-lactic acid) (Hadlock, Sundback et al. 2000) do not promote nerve regeneration as well as autografts, they do promote better regeneration than unseeded NGCs. Alternatively, biologically-derived grafts seeded with SCs may be used to promote nerve regeneration. Seeding nerve allografts (ANGs and allografts) with SCs has been found to improve regeneration compared to unseeded grafts in both rat and swine models. Experimental evidence suggests that following transplantation, cultured SCs survive and promote regeneration by increasing the synthesis of cell-cell adhesion molecules, such as N-cadherin and N-CAM/L1, growth factors, such as NGF, GDNF, and BDNF, and also provide the myelin sheath for regenerating axons (Gulati 1988; Levi and Bunge 1994; Levi, Guenard et al. 1994; Fansa, Keilhoff et al. 1999). To produce a sufficient number of SCs for transplantation, mitogens can be used to expand SCs *in vitro* (Morrissey, Kleitman et al. 1991; Bunge 1993; Levi, Bunge et al. 1995). However it is important to note that expanded human and rat SCs failed to demonstrate tumor formation when transplanted into rodents after mitogen addition is removed (Emery, Li et al. 1999; Ogden, Feng et al. 2000). Therefore, transplantation of SCs into NGCs or biological grafts aids in successful peripheral nerve regeneration. But, the regeneration in grafts does not compare to the clinical gold standard, autografts, suggesting that the SCs may lack certain environmental cues to further differentiate and promote myelination axons to match the regeneration of the autograft.

1.5   Environmental Cues

Typically, after denervation, SCs dedifferentiate into an immature state, where they secrete increased amounts of ECM molecules and neurotrophic factors (NGF, BDNF, GDNF) to guide regenerating axons from the proximal to the distal stump (Bunge 1993; Jessen and Mirsky 2005). Once the axons start regenerating, cues from the environment must promote the redifferentiaton into mature SCs to remyelinate the regenerating axons. The increased amount of ECM molecules, such as collagen, laminin, and fibrin, interact with the SCs at the injury site. SC interactions with the ECM promotes migration of SCs, SC proliferation, and intracellular signaling (Bixby, Lilien et al. 1988). The delivery of neurotrophic factors, such as NGF and GDNF, at the injury site promotes axonal regeneration (Lee, Yu et al. 2003; Wood, Moore et al. 2009; Wood, Macewan et al. 2010). Therefore it is possible that the interaction between the GFs and SCs at the injury site may promote redifferentiation of the SCs and remyelination of the axons (Chan, Cosgaya et al. 2001; Wong, Henley et al. 2002; Iwase, Jung et al. 2005). Lastly, small molecules, such as neurotransmitters may have effects on reinforcing SC phenotype. For example, the presence of acetylcholine (Ach) at the neuromuscular junction (Anderson and Stevens 1973) may aid in the reinforcement of the mature motor phenotype. These different environmental cues will be discussed in more detail in the following sections.

1.5.1 Extracellular Matrix (ECM)

After injury, the assembly and maintenance of the ECM is necessary to promote increased migration and proliferation of SCs. Interactions with SCs also help with remyelination of the axons during regeneration. In the PNS, the basal lamina (laminin, collagen IV) is produced by the axon-associated SCs, and it surrounds the external surface of

the SC-axon units. To understand the importance of the basal lamina, experiments studying the effects of the basement membrane on SC myelination and ensheathement of axons have been performed *in vitro*. SCs myelinate axons when serum and ascorbic acid is added to the medium (Eldridge, Bunge et al. 1987). If basement membrane assembly is prevented by the use of a serum-free medium (Moya, Bunge et al. 1980) or myelination is blocked by inhibiting collagen hydroxylation with cis-hydroxyproline (Eldridge, Bunge et al. 1988), SCs remain in a non-myelinating state. But with the addition of exogenous basement membrane (Elsdale and Bard 1972; Kleinman, McGarvey et al. 1986), SCs myelinate the axons in serum free media or enhance myelination in serum and ascorbic acid containing cultures (Eldridge, Bunge et al. 1989). However, in the absence of neurons, the necessary axonal signaling to SCs to form basement membrane is lost, and the addition of basement membrane proteins to these cultures promotes proliferation rather than differentiation and myelination (Cornbrooks, Carey et al. 1983; McGarvey, Baron-Van Evercooren et al. 1984; Baron-Van Evercooren, Gansmuller et al. 1986). From these studies, it can be clearly seen that interactions with the basement membrane are necessary to promote differentiation of SCs and myelination of axons. Further research has shown that signals generated by interactions between SCs and the basement membrane may contribute to the regulation of glycolipid synthesis, which may affect the cell morphology, proliferation, and possibly differentiation of SCs (Farrer and Quarles 1996).

It has been shown that SCs migrate out of the distal stump post injury. In a previous study, time lapse microscopy showed the migration of two distinct SC populations from explanted injured nerves *in vitro*. Single SCs emerged from the nerve randomly, but eventually arranged themselves in an organized array. In the second group, a more coordinated movement was observed where one SC led and another followed behind it.

These possible migratory patterns of SCs may contribute to regeneration of nerves due to the orderly alignment of the SCs (Crang and Blakemore 1987). Since SCs exhibit these migratory and alignment patterns, researchers have studied how ECM molecules such as laminin may affect the alignment of SCs *in vitro* (Thompson and Buettner 2001). These results demonstrate that the interaction of SCs on micropatterned laminin surfaces aligns SCs, and thus promotes neurite outgrowth of axons in the same direction as the SCs (Thompson and Buettner 2006). Alternatively, it has been shown that SCs migrate into magnetically aligned type I collagen gels, and align themselves in patterns reminiscent of the bands of Bungner in the presence of serum (Dubey, Letourneau et al. 1999) to guide neurite extension. Together, these studies demonstrate that SC interaction with patterned ECM molecules initiates SC alignment and formations similar to the bands of Bunger, which are necessary to guide regenerating axons to the distal stump.

These interactions between SCs and ECM molecules may not only promote differentiation of SCs and remyelination of axons, but also promote SC alignment to guide axonal regeneration. Using this information and the characteristics of the interactions, better SC transplantation therapies can be designed to increase the rate and efficiency of peripheral nerve regeneration. SC alignment in response to collagen or laminin present within the injury site or in nerve grafts, may aid in guiding the regenerating axons from the proximal to the distal stump.

1.5.2 Growth Factors (GFs)

GFs are not only necessary to maintain neuronal survival but are upregulated to promote neural regeneration post-injury (Costigan, Befort et al. 2002). These factors typically exert their effect by binding to cell-surface receptors, which leads to many

downstream effects. SCs are also affected by GFs, such as NGF and GDNF, in the environment. SCs have surface receptors for both NGF and GDNF, which may affect differentiation into the mature myelinating phenotypes (Chan, Cosgaya et al. 2001; Wong, Henley et al. 2002; Iwase, Jung et al. 2005).

NGF, the first discovered neurotrophin, was found in mouse sarcoma and identified based on its effects on chick embryo ganglia (Levi-Montalcini and Hamburger 1951). The processed and biologically active form of NGF (β-NGF) is a dimer with 3 disulfide bonds to stabilize itself (McDonald, Lapatto et al. 1991; Sofroniew, Howe et al. 2001). NGF was reported to protect neurons from injury-induced death in sciatic nerves due to complete nerve transection (Otto, Unsicker et al. 1987). The addition of NGF to saline-filled conditions has shown to increase myelinated axons in the regenerating nerve (Rich, Alexander et al. 1989). The increase in myelinated axons suggests that the presence of NGF in the silicone conduits promoted the differentiation of the SCs to increase myelination. A known surface receptor of immature SCs is the $p75^{NTR}$, which is also an NGF receptor (Tomita, Kubo et al. 2007). The activation of the $p75^{NTR}$ promotes cell survival, apoptosis, and differentiation through a variety of signaling pathways (Hirata, Hibasami et al. 2001; Segal 2003; Nicol and Vasko 2007). In previous studies, it has been shown that the differentiation of SCs may also be due to the elevation of intracellular cyclic adenosine monophosphate (cAMP) (Salzer, Williams et al. 1980; Morgan, Jessen et al. 1991; Monje, Soto et al. 2010). It has been hypothesized that the stimulation of $p75^{NTR}$ may increase the intracellular levels of cAMP, which may further contribute to the redifferentiation of the SCs (Monje, Soto et al. 2010)and the myelination of regenerating axons.

Similarly, SCs also express receptors for GDNF, which was the first discovered of the GDNF family of ligands (Lin, Doherty et al. 1993; Baloh, Enomoto et al. 2000). GDNF

has primarily been identified for the survival of dopaminerginic and motor neurons (Lin, Doherty et al. 1993; Henderson, Phillips et al. 1994; Hoke, Cheng et al. 2000). SCs express glycosylphosphatidylinositol (GPI)-anchored family receptor (GFR)α1, which is a receptor for GDNF (Naveilhan, ElShamy et al. 1997) and neural cell adhesion molecule (NCAM), which acts as a co-receptor that facilitates GDNF-mediated signaling in SCs (Iwase, Jung et al. 2005). Exogenous GDNF has been shown to promote SC proliferation and myelination of normally unmyelinated small axons (Hoke, Ho et al. 2003). Mechanistically, GDNF has been shown to activate pathways in SCs implicated in cell migration, proliferation, differentiation and GF production (Morgan, Jessen et al. 1991; Lang, Gesbert et al. 1996; Kim, DeClue et al. 1997; Klemke, Cai et al. 1997; Grimm, Holinski-Feder et al. 1998; Verity, Wyatt et al. 1998; Meintanis, Thomaidou et al. 2001; Ellerbroek, Wennerberg et al. 2003; Kinameri and Matsuoka 2003; Iwase, Jung et al. 2005). In addition, it has been hypothesized that activation of the GDNF signaling pathway may also increase the intracellular levels of cAMP, which may lead to differentiation of SCs (Monje, Soto et al. 2010). *In vivo*, the initial presence of GDNF at the injury site can guide axons from the proximal to the distal stump. As the axons regenerate, GDNF may signal to the SCs to differentiate and remyelinate the axons.

1.5.3 Acetylcholine (Ach)

As mentioned earlier, SCs have different phenotypes based on their nerve source. Previous studies have shown that the SCs derived from the cutaneous branch of the rat femoral nerve and SCs derived from ventral root differentially express certain growth factors (Hoke, Redett et al. 2006). These specific phenotypes may influence the regenerating pathways of axons after injury. Much research has been conducted to regenerate motor

axons and reinnervate muscle to enhance functional recovery. Ach, a neurotransmitter found at the NMJ, may promote differentiation of SCs into a motor phenotype, which may in turn influence the pathway a regenerating motor axon chooses.

Ach is present in both the CNS and the PNS and behaves as an excitatory neurotransmitter (Anderson and Stevens 1973). In the PNS, Ach binds to Ach receptors on skeletal muscle fibers and activates muscle contraction. It opens ligand-gated sodium channels in the cell membranes and initiates a sequence of steps that help contract the muscles (Macintosh 1941). Because Ach is released at the NMJ, possible interaction with the SCs may promote the expression of motor-specific markers. Additionally, Ach present at growth cones of regenerating axons may also contribute to the differentiation of SCs. SCs express muscarinic receptors M1 though M4 for acetylcholine with M2 expressed at the highest level (Loreti, Vilaro et al. 2006). Early work done with Ach and SCs showed that Ach does not have an effect on the viability of the SCs at some concentrations (Salzer and Bunge 1980). However, interaction with the receptors may promote the differentiation and expression of motor markers in SCs. In a recent *in vivo* study, it was shown that blocking of the Ach receptors during motor nerve regeneration prevented the regeneration of motor axons into the distal nerve stump suggesting that interaction of Ach with SC receptors is necessary for motor nerve regeneration (Vrbova, Mehra et al. 2009). The M1 and M3 receptors have been shown to promote the increase in intracellular cAMP and M2 and M4 receptors prevent the increase of cAMP (Felder 1995). Thus the interaction of Ach with these receptors may increase intracellular levels of cAMP and promote the differentiation of the SCs and the expression of motor specific markers in SCs, which may influence the specific regeneration of motor axons. Further studies need to be done to evaluate the effects of Ach on the differentiation and motor marker expression in motor SCs.

1.6   Concluding Remarks

Previously it has been shown that SCs derived from the cutaneous (sensory) branch of the rat femoral nerve and the ventral root exhibit phenotypic differences (Hoke, Redett et al. 2006). This study identified specific genes that are differentially expressed in sensory and motor SCs. In this study, it was also shown that SCs respond differently to denervation and reinnervation by sensory or motor axons. These differences among gene expression patterns persisted despite prolonged contact with axons of the opposite phenotype. Thus, these findings support the hypothesis that the differences in phenotype of SCs may influence the pathway regenerating axon chooses, either motor or sensory. However, for phenotype-specific SCs to be transplanted at the injury site, SCs need to be expanded in culture. To ensure that the phenotype of SCs is preserved in culture, SC gene expression patterns need to be monitored over the expansion period. Therefore, this thesis work first derived SCs from motor and sensory nerves and specific genes that are differentially upregulated in each branch were identified.

The rat femoral nerve was chosen as the source of the SCs due to its bifurcation into the quadriceps (motor) branch and the cutaneous (sensory) branch. Although these nerve lengths are small, they provide a pure population of motor and sensory SCs for evaluation (Brushart 1993). For this thesis work, the gene expression patterns of SCs derived from these nerves were evaluated under different conditions. The first study focused on identifying genes that are differentially expressed in each branch. Using these genes, the expression patterns were monitored as the SCs were expanded in culture. The second and third studies were conducted to understand the effects of environmental cues on the differentiation into mature motor and sensory SCs. In the second study, the SCs were

transplanted in cold preserved acellular nerve grafts (CP-ANGs) to understand the effects that ECM would have on SC phenotype and GF expression. The nerve grafts with SCs were then used to treat a 14 mm rat sciatic nerve gap injury. In the third study, SCs were treated with media supplemented with GFs, NGF and GDNF, and the neurotransmitter Ach (motor SCs only) to understand the effects that these cues have on the expression of differentiation and phenotype-specific markers. Overall, this thesis work serves to evaluate possible environmental cues that affect the behavior of SCs *in vitro*, and thus may aid in the designing of cell transplantation therapies to promote motor or sensory nerve specific regeneration *in vivo*.

Chapter 2

Differential Gene Expression in Motor and Sensory Schwann Cells in the Rat Femoral Nerve[*]

2. 1   Abstract

Phenotypic differences in Schwann cells (SCs) may help guide axonal regeneration down motor or sensory specific pathways following peripheral nerve injury. The goal of this study was to identify phenotypic markers for SCs harvested from the cutaneous (sensory) and quadriceps (motor) branches of the rat femoral nerve and to study the effects of expansion culture on the expression patterns of these motor or sensory phenotypic markers. RNA was extracted from SCs harvested from the motor and sensory branches of the rat femoral nerve and analyzed using Affymetrix Gene Chips© (Rat Genome 230 v 2.0 Array A). Genes that were upregulated in motor SCs compared to the sensory SCs or vice versa were identified, and the results were verified for a subset of genes using quantitative real time polymerase chain reaction (qRT-PCR). The expression levels of the "phenotype-specific" genes were then evaluated in SC expansion cultures at various timepoints over 30 days by qRT-PCR to determine the effect of expansion on SC phenotype. Expression levels of the phenotype-specific genes were significantly altered after expansion culture for both the motor and sensory markers compared to fresh nerve tissue. These results indicate that both motor and sensory SC gene expression patterns are disrupted during expansion *in vitro* and may affect the ability of SCs to express phenotype specific genes after transplantation.

2.2 Introduction

Peripheral nerve injury (PNI) due to a complete nerve transection results in a loss of function. Ideally, the two severed ends of the nerves can be rejoined using a direct end to end coaptation. However, in larger nerve gap injuries, a direct coaptation can introduce unnecessary tension that may impede regeneration. To prevent tension and to bridge the nerve defect, an autograft can be used to provide extracellular matrix (ECM) molecules and growth factors (GFs) to promote regeneration of axons across the nerve gap. Although the nerve autograft remains the gold standard of care, this method has limitations including donor site morbidity, lack of sufficient donor tissue, and size mismatches at the injury site (Schmidt and Leach 2003; Burnett and Zager 2004). Currently, investigators are searching for alternative therapies to bridge nerve gaps following injury, such as acellular nerve grafts (ANGs).

ANGs have been used to support the growth of regenerating axons from the proximal nerve into the distal stump (Hare, Evans et al. 1993; Levi, Evans et al. 1994; Hare, Evans et al. 1995). Compared to nerve conduits, ANGs support superior nerve regeneration because they contain an intact microstructure consisting of endoneurial tubes and ECM that supports regenerating axons (Johnson, Duhamel et al. 1982; Sondell, Lundborg et al. 1998; Whitlock, Tuffaha et al. 2009). However, the regenerative capacity of ANGs is still inferior to autografts because they lack SCs (Whitlock, Tuffaha et al. 2009). SCs provide GFs and ECM to promote neuronal survival and axonal regeneration (Bunge 1993; Reynolds and Woolf 1993; Frostick, Yin et al. 1998; Nagarajan, Le et al. 2002; Brenner, Lowe et al. 2005). Addition of SCs to ANGs has been proposed as a method to enhance their regenerative capacity.

When given equal access to motor and sensory pathways, the motor axon tends to regenerate down the motor pathway. This phenomenon, preferential motor reinnervation (PMR), may be influenced by trophic support from the end organs (with muscle greatly outweighing skin in PMR) or the SCs present within terminal nerve pathways (Brushart 1988; Brushart 1993; Madison, Archibald et al. 1996; Madison, Archibald et al. 1999; Madison, Robinson et al. 2007; Madison, Sofroniew et al. 2009). Previous studies have shown that sensory axons may also regenerate preferentially down sensory pathways (Hoke, Redett et al. 2006). In addition to guiding the regeneration of axons, SCs derived from the cutaneous branch of the rat femoral nerve and the SCs derived from ventral root of the sciatic nerve exhibit phenotypic differences that may influence the regenerating pathway (Hoke, Redett et al. 2006). After denervation, SCs de-differentiate into an immature state (Mirsky and Jessen 1996) and secrete GFs and ECM to enhance regeneration (Bunge, Bunge et al. 1986; Bunge 1993; Bunge 1994). Although SCs de-differentiate, they may retain a "phenotypic memory" that allows them to re-differentiate into their original phenotype during regeneration, as evidenced by different expression profiles observed by motor and sensory SCs even after prolonged contact with axons of the opposite phenotype (Hoke, Redett et al. 2006) .

Understanding how SC gene expression changes during de-differentiation and following transplantation could enable motor or sensory specific nerve regeneration using ANGs seeded with phenotype specific SCs. In this study, a set of phenotypic markers were identified for the motor and sensory SCs, harvested from the motor and sensory branches of the rat femoral nerve, using Affymetrix Gene Chips© and quantitative real time polymerase chain reaction (qRT-PCR) and to study the effects of expansion culture conditions on the

SC gene expression profiles. The results indicate that both motor and sensory SCs have unique phenotypes that are disrupted when expanded *in vitro*.

2.2 Materials and Methods

2.3.1 RNA preparation

Male Lewis rats (400-500 grams) were anesthetized to undergo bilateral harvest of the sensory and motor branches of the femoral nerve and were then euthanized. The nerves were stored at -80°C in RNA*later*® solution (Ambion, Austin, TX). Total RNA was extracted from homogenized nerves using an acid phenol extraction (TRIzol Reagent, Invitrogen, Carlsboro, CA). The aqueous layer was collected, and the samples were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). The presence of the RNA was assessed by electrophoresis using 2% agarose gels after running reverse transcriptase PCR with a β-actin primer. To verify that the mRNA extracted from the nerves met the quality standards for further experiments, the mRNA concentration (0.2 – 0.6 µg/µL) was determined by measuring the absorbance at 260 nm and the quality was verified using an absorbance ratio of A260/A280. The ratio threshold was held at 1.8, which signifies a high purity of RNA in the sample (Wilfinger, Mackey et al. 1997). The extracted RNA was used for further experiments. Since the majority of the nerves are composed of SCs (~90%) (Oda, Okada et al. 1989), the harvested RNA was assumed to be representative of the SC RNA present in fresh nerve tissue.

2.3.2 Gene Chips

The RNA (10 nerves pooled from different male Lewis rats per sample) required for gene chip analysis was prepared according to the standard protocol provided by the Siteman Cancer Center GeneChip Facility at Washington University and run in triplicate. Purified total RNA (10 μg) was spiked with a set of four synthetic, polyadenylated, and bacterial transcripts (Lys, Phe, Thr, and Trp) diluted to defined copy numbers. Oligonucleotide probes for these transcripts were present on all Affymetrix GeneChips, thus monitoring the expression level of these internal standards provided an indication of the total technical variability associated with the experiment. Spiked RNA was converted to cDNA, purified, and then used as a template for *in vitro* transcription of biotin-labeled antisense RNA. All protocols were performed as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Each biotinylated antisense RNA preparation (20 μg) was fragmented, assessed by gel electrophoresis, and placed in hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre). Samples were hybridized to Affymetrix Rat GeneChip® Rat Genome 230 2.0 Array A for 16 h ($n = 3$ chips for each condition). GeneChips were washed and stained using the instrument's standard Eukaryotic GE Wash 2 protocol, using antibody mediated signal amplification. The images from the scanned chips were processed using Affymetrix Microarray Analysis Suite 4.0. Genes that did not hybridize to the probe for any sample were excluded from the analysis.

The results obtained from the gene chips were analyzed using the exclusion criterion (Costigan, Befort et al. 2002) in combination with Spotfire DecisionSite 9.0 and Microsoft Excel. If the gene was not present in at least two of the chips in each group, then it was excluded from the gene set. The detection signal was then z-score normalized and statistical analysis (ANOVA) paired with a t-test was performed to compare the detection signals of

the sensory nerves and the motor nerves, and the gene was excluded if the p-value was greater than 0.05, which is the threshold of significance. The remaining data was imported to Microsoft Excel. Genes that had a mean value varying by less than two-fold between sensory and motor or with a standard deviation between replicates in the same group that was greater than 35% of the mean were excluded from the analysis.

2.3.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

cDNA was synthesized from the isolated RNA using the QuantiTect® Reverse Trascription Kit (Qiagen). Using the QuantiTect® SYBR® Green PCR mastermix (Qiagen) in combination with gene specific QuantiTect® primer assays, qRT-PCR was performed using an Applied Biosystems 7000 Real-Time PCR thermocycler. The genes studied included vascular endothelial cell growth factor (VEGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), pleiotrophin (PTN), glial-derived neurotrophic factor (GDNF), myelin basic protein (MBP), protein kinase C iota (PRKCi), neuroligin 1 (NLGN1), and neurofilament (NEFL). The primers for those mentioned proteins were added to the cDNA for each sample present for the motor and sensory nerves. The qRT-PCR was conducted using the following conditions: (1) 50°C for 2 min (2) 95°C for 15 min, and (3) forty cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds (Gaumond, Tyropolis et al. 2006). Target genes were normalized to an internal control (β-actin) to account for the variation in cDNA concentration between samples, and appropriate negative control samples were present (no template control). The QuantiTect® primer assays are validated to have a PCR efficiency of 100%. To estimate the mRNA concentrations, the differences in gene expression levels between two different samples were

31

calculated using the comparative delta crossover threshold (C_t) method (Livak and

Schmittgen 2001; Pfaffl 2001; Schmittgen and Livak 2008).

2.3.4 SC Culture Preparation for Time Study

SC cultures were prepared as previously described (Raff, Abney et al. 1978; Pruss

1982). Briefly, the sensory and motor branches of the rat femoral nerve were harvested and

placed in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Collagenase I (1%) (Fisher,

Pittsburgh, PA) and trypsin (2.5%) (Invitrogen) were added to the fascicles and incubated

for 30 min at 37°C. After centrifugation at 130 x g for 5 min, the pellet was washed with

Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-

activated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% antibiotic

antimycotic (ABAM, Invitrogen). The cells were then seeded on 24 well plates coated with

poly-L-lysine (pLL) (Sigma-Aldrich). Tissue culture plates were prepared by coating with 1

mL 0.01% pLL in sterile water and washing twice with sterile water. On day 2 of culture, 10

μM Cytosine-beta-arabino furanoside hydrochloride (Ara-C) (Sigma-Aldrich), was added to

cultures along with the media containing DMEM, FBS and ABAM. On day 6, the

fibroblasts were complement-killed using an anti-Thy 1.1 antibody (1:40 dilution in media,

Serotec, Raleigh, NC) and guinea pig complement (1:4 dilution in media, Sigma-Aldrich).

On subsequent days the culture media was supplemented with 2 μM forskolin (Sigma-

Aldrich), and 20 μg/mL pituitary extract (PE) (Biomedical Tech, Inc., Stoughton, MA).

RNA was extracted from Days 1, 3, 7, 14 and 30 using an acid-phenol extraction and was

purified using an RNeasy Mini Kit (Qiagen). qRT-PCR was performed for each gene at each

time point and compared to the gene expression of each gene in freshly harvested femoral

motor and sensory nerves.

2.3.5 Statistical Analysis

Statistical analyses were performed using SigmaStat 3.0 (Systat Software, San Jose, CA), and all data were evaluated with one-way analysis of variance (ANOVA), followed by a Scheffe's F test for comparisons between groups when significance (p<0.05) was present. All results are reported as mean ± standard deviation.

2.4 Results

2.4.1 Gene Chips

The differences in gene expression between SCs in the motor and sensory branches of the rat femoral nerve were evaluated using Affymetrix gene chips and qRT-PCR. Similar to findings in literature, we assumed that the majority of the RNA harvested from these nerves (~90%) was from SCs (Oda, Okada et al. 1989). RNA was extracted from the motor and sensory branches of fresh rat femoral nerves, and analyzed using gene chips to obtain a set of genes that were differentially upregulated in the motor SCs or sensory SCs when compared to each other. Using a stringent criteria, which has been shown to generate the lowest number of false positives (Costigan, Befort et al. 2002), ~100 genes were identified to be differentially upregulated in either the sensory (76) or motor (23) branches of the femoral nerve (data not shown).

In motor SCs, a subset of the upregulated genes was identified to be involved in different functions related to motor nerve myelination and signaling (Table 2.1). For example, NEFL has been shown to be an important factor in the myelination of the motor

axons (Roxanne et. al 2003, Roberson et. al 1992), which suggests that NEFL may be a good marker for motor SCs. In SCs, PRKCi may act through the p75^{NTR} activation pathway to promote survival or apoptosis (Mamidipudi et. al 2002). PRKCi has also been shown to interact with Rab2 to promote vesicle budding for exosome formation to facilitate intercellular signaling between SCs and axons (Tisdale 2000; van Niel, Porto-Carreiro et al. 2006). The increased expression of PRKCi in the motor SCs may be attributed to having more signaling between the motor SCs and axons than the sensory SCs due to the higher number of motor neurons (and thus motor axons and motor SCs) present in the peripheral nervous system (PNS).

A subset of the genes identified to be differentially upregulated in the sensory SCs has been shown to be involved in promoting sensory nerve myelination and maturation (Table 2.2). Neuroligin 1 (NLGN1) is a component of the myelin sheath made by SCs (Song, Ichtchenko et al. 1999; Jahn, Tenzer et al. 2009). Because the expression of NLGN1 in SCs is increased during sensory nerve depolarization and signal conduction in the SC-associated axons, NLGN1 makes a good candidate as a sensory SC marker (Biswas et al. 2010). Although MBP is present in both motor and sensory SCs and promotes myelination (Eylar, Brostoff et al. 1971) of axons, increased intracellular progesterone in SCs, (Guennoun, Benmessahel et al. 2001; Robert, Guennoun et al. 2001), increases the expression of MBP in sensory SCs, thus making MBP a good marker for sensory SCs.

Table 2.1: Genes that are upregulated in the motor branch of the femoral nerve versus sensory branch. $M-$ average signal intensity in motor nerve group, $S-$ average signal intensity in sensory nerve group ($n = 3$)

Gene name	Gene Common name	Accession Number	Fold Difference M/S	std dev/average
Peripheral myelin protein 2	Pmp2	AW533483	5.62	0.19
Four and a half LIM domains 1	Fhl1	BI298356	4.83	0.22
Gap junction membrane channel protein beta 2	Gjb2	AI179953	3.48	0.21
Neurofilament, light polypeptide [a]	Nefl	NM_031783	3.46	0.02
Prostaglandin D2 synthase	Ptgds	J04488	2.33	0.1
Ubiquitin carboxy-terminal hydrolase L1	Uchl1	NM_017237	2.28	0.03
Calsequestrin 2	Casq2	NM_017131	2.07	0.31
Amphiphysin 1	Amph1	NM_022217	2.05	0.28
Fucosidase, alpha-L-2, plasma	Fuca2	BM389993	2.04	0.11
Tubulin, beta 2b	Tubb2	X03369	2.01	0.07
Protein kinase C, iota [a]	Prkci	AB020615	2.00	0.11

[a] indicates genes used for further studies

Table 2.2: Genes that are upregulated in the sensory branch of the femoral nerve versus motor branch. M – average signal intensity in motor nerve group, S – average signal intensity in sensory nerve group ($n = 3$)

Gene name	Gene common name	Accession Numbers	Fold Difference S/M	std dev/average
Neuroligin 1[a]	Nlgn1	BF400127	3.36	0.34
Ankyrin 3, epithelial	Ank3	AJ428573	2.52	0.03
S100 calcium binding protein A9 (calgranulin B)	S100a9	NM_053587	2.23	0.14
microtubule-associated protein tau	Mapt	BE107978	2.22	0.33
scavenger receptor class B, member 1	Scarb1	NM_031541	2.21	0.07
L1 cell adhesion molecule	L1cam	NM_017345	2.19	0.09
Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	BE102996	2.17	0.24
Myelin basic protein[a]	Mbp	BE109730	2.14	0.12
Platelet derived growth factor receptor, beta polypeptide	Pdgfb	AI071374	2.13	0.06
Neural cell adhesion molecule 1	Ncam1	AI576209	2.05	0.06
nuclear receptor subfamily 4, group A, member 2	Nr4a2	U72345	2.04	0.23

[a] indicates genes used for further studies

2.4.2 Verification of genes using qRT-PCR

For further analysis, a set of genes was selected from the gene chip analyses that have been shown in previous studies to be involved in either sensory or motor function. development and growth: neuroligin 1 (NLGN1, sensory), myelin basic protein (MBP, sensory), protein kinase C iota (PRKCi, motor), and neurofilament (NEFL, motor) (Roberson, Toews et al. 1992; Tisdale 2000; Robert, Guennoun et al. 2001; Biswas, Reinhard et al. 2010). Along with these markers, genes that were previously shown to be differentially expressed in motor and sensory SCs were also used for analysis: VEGF (motor), PTN (motor), GDNF (sensory), BDNF (sensory), and NGF (similar expression in motor and sensory) (Hoke, Redett et al. 2006). The expression levels in fresh nerve tissue were evaluated for the selected genes using qRT-PCR to verify that the chosen genes agreed with the trends found in the gene chip analysis and in literature. PRKCi and NEFL were upregulated in motor SCs compared to sensory SCs (Figure 2.1), which agreed with the trends observed with the gene chip analysis. Previously identified markers from literature were also analyzed by qRT-PCR (Figure 2.2), and those results showed similar trends to those found in the literature. The genes VEGF and PTN were upregulated in the motor SCs compared to the sensory SCs, BDNF was downregulated in motor SCs compared to the sensory SCs, and NGF was expressed in similar levels in both types of SCs. These results suggest that we can use these gene markers to identify the phenotype of the SCs because they correlated well with results from the gene chips and literature.

Genes from Gene Chip

Upregulated in the motor nerve
in gene chip analysis

*p = 0.02

Upregulated in the sensory nerve in
gene chip analysis

*p = 0.01

p = 0.26   p = 0.20

NLGN1   MBP   PRKCi   NEFL

mRNA fold difference in fresh motor nerve compared
to fresh sensory nerve
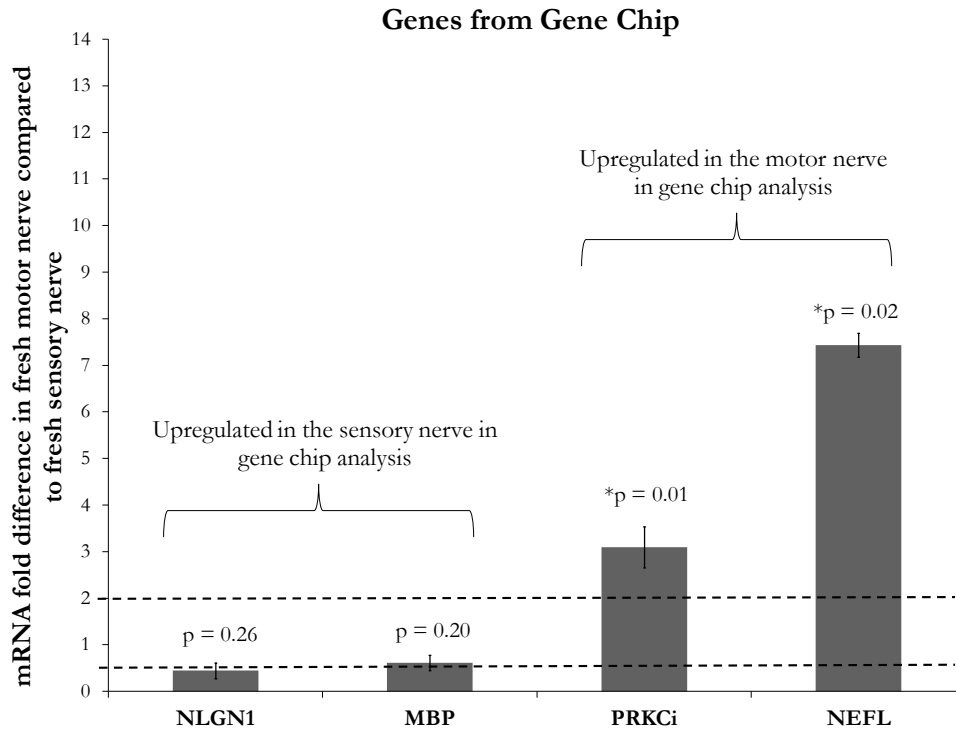
Figure 2.1. Verification of gene chip trend with qRT-PCR. The expression level of genes that were identified to be differentially expressed in the motor and sensory branches of the rat femoral nerve was determined by qRT-PCR. The values were normalized to β-actin, and the fold difference versus sensory nerve was calculated. PRKCi and NEFL are upregulated in the motor branch similar to the gene chip results. Error bars represent the standard deviation (*n=3*). *The dotted line at 2 is the threshold value for up regulation, and the dotted line at 0.5 is the threshold for down regulation. * denotes p-values are the significance levels between the $\Delta C_{t\ (motor - \beta\text{-actin})}$ and $\Delta C_{t\ (sensory - \beta\text{-actin})}$*

Genes from Literature

Y-axis: mRNA fold difference in fresh motor nerve compared to fresh sensory nerve

Upregulated in the motor nerve in literature

*p = 0.004

Expressed in similar levels

*p = 0.01

Upregulated in the sensory nerve in literature

p = 0.33

*p = 0.02   p = 0.47

X-axis categories: BDNF, GDNF, NGF, VEGF, PTN
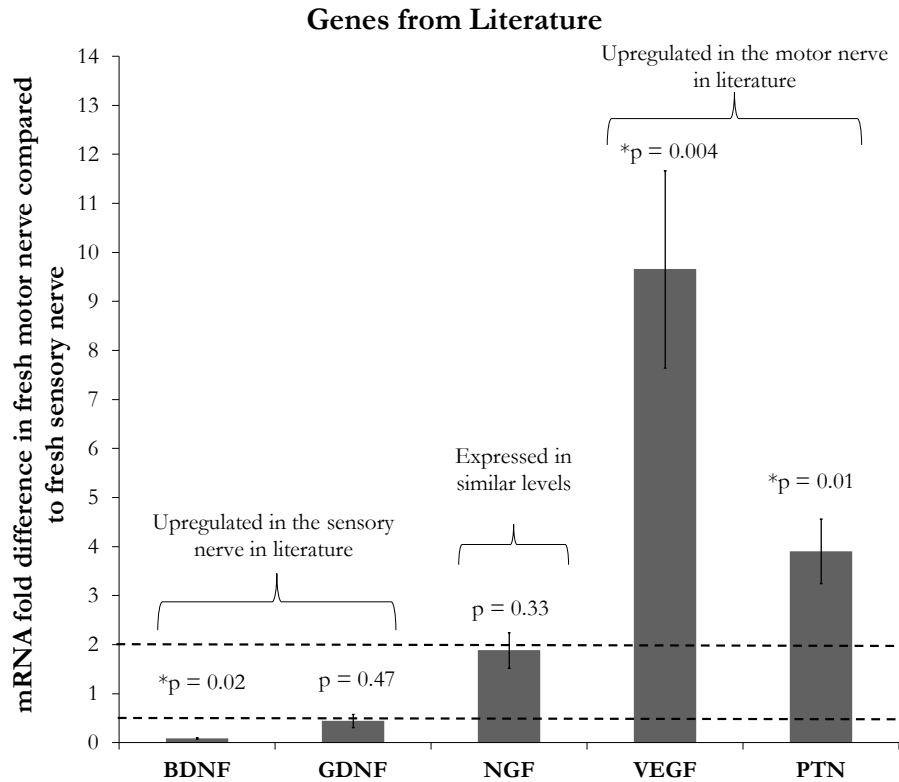
Figure 2.2. Expression of genes previously identified as markers for sensory and motor SCs. The expression level of genes that were reported in the literature to be differentially regulated in the motor and sensory SCs (Hoke, Redett et al. 2006) was determined by qRT-PCR. The values were normalized to β-actin, and the fold difference versus sensory nerve was calculated. BDNF is upregulated in the sensory branch, NGF is similarly expressed in both branches, and VEGF and PTN are upregulated the motor branch. Error bars represent the standard deviation (*n=3*). *The dotted line at 2 is the threshold value for up regulation, and the dotted line at 0.5 is the threshold for down regulation. * denotes p-values are the significance levels between the $\Delta C_{t\ (motor\ -\ \beta\text{-}actin)}$ and $\Delta C_{t\ (sensory\ -\ \beta\text{-}actin)}$*

2.4.3 Gene expression in SCs in vitro

To evaluate the effect of expansion culture on differential gene expression, SCs from the motor and sensory branches of the femoral nerve were harvested and cultured for 30 days. Fibroblasts were eliminated from the culture using Ara-C to inhibit the proliferation of the cells (Ogbomo, Michaelis et al. 2008) and the cells were allowed to recover for 4 days before the remaining fibroblasts were killed using complement. SC RNA was collected at days 0, 1, 3, 7, 14, and 30 (Table 2.3), and the expression levels of the genes (Table 2.4) were analyzed using qRT-PCR compared to expression in fresh nerve tissue (day 0). In these studies, a value of two or greater was selected as the minimum criteria for a significant difference in expression levels between groups (Hoke, Redett et al. 2006).

Table 2.3: The different conditions at which the RNA was extracted for qRT-PCR

Day	RNA Condition
0	From freshly harvested nerve tissue
1	From cells plated for 24 hours on pLL coated plates
3	From cells recovering from the Ara-C treatment
7	From cells recovering from complement killing of fibroblasts
14	From cells at ~30% confluence
30	From cells at ~80% confluence

Table 2.4: The genes chosen from the gene chip analysis and literature for further analysis

Gene	Gene Common Name	Differentially upregulated or similarly expressed in motor or sensory SCs	Function in the Nervous system
NEFL	Neurofilament Light Peptide	Motor (Gene Chips)	Helps with the axonal growth and myelination (Roberson et al. 1992)
PRKCi	Protein Kinase C iota	Motor (Gene Chips)	Regulates intercellular signaling between SCs and axons (Tisdale 2000; van Niel et al. 2006)
NLGN1	Neuroligin 1	Sensory (Gene Chips)	Associated with the localization in the postsynaptic compartment of excitatory synapses (Biswas et al. 2010; Scheiffele et al. 2000)
MBP	Myelin Basic Protein	Sensory (Gene Chips)	Responsible for the myelination of nerves in the nervous system (Eylar et al. 1971)
VEGF	Vascular Endothelial Growth Factor	Motor (Literature)	Creates new blood vessels during adult nervous system development (Rosenstein et al. 2008)
PTN	Pleiotrophin	Motor (Literature)	Neurite outgrowth promoting factor (Jin et al. 2009)
NGF	Nerve Growth Factor	Similar (Literature)	Important for the growth, maintenance and survival of certain target neurons (Chan et al. 2001)
GDNF	Glial Derived Neurotrophic factor	Sensory (Literature)	Promotes the survival and differentiation of dopaminergic neurons (Iwase et al. 2005)
BDNF	Brain-derived Neurotrophic factor	Sensory (Literature)	Helps with the support, survival, growth, differentiation of new neurons and synapses (Chan et al. 2001)

Those genes that showed significant changes in gene expression over 30 days were MBP, NEFL, PTN and PRKCi (Figure 2.3). MBP is differentially upregulated in the sensory branch of the femoral nerve in fresh nerve tissue. The expression in the sensory SCs increases over the course of the study as does the expression in the motor SCs. On Day 1, there is a difference in the expression of the MBP, which is greater in motor SCs versus sensory SCs. Eventually the gene is expressed at similar levels until day 30 when the expression in the sensory SCs increases and expression in motor SCs drops (Figure 2.3A). Of the other genes that were evaluated, NEFL, PTN and PRKCi (all motor markers) showed changes in the expression over 30 days. NEFL (Figure 2.3B) expression decreased in the motor SCs but in the sensory SCs there was a 40 fold increase over the fresh tissue after 30 days. PTN expression levels were upregulated in the sensory SCs and increased to a maximum expression at day 14 with a 35 fold increase when compared to the expression in the fresh tissue (Figure 2.3C). In the motor SCs, the PTN gene was downregulated over 30 days of expansion culture. PRKCi showed similar expression levels when compared to the fresh tissue of the motor nerve, but the expression levels in the sensory SCs increased approximately 8 fold when compared to the fresh sensory nerve tissue (Figure 2.3D). The gene expression for the remaining five genes, GDNF, BDNF, NGF, VEGF, and NLGN1 showed no significant increases in expression because all the mRNA fold difference values were below the value of two (data not shown). From these results, we can see that the phenotypic expression of these genes in motor or sensory SCs is significantly affected when SCs are expanded *in vitro.*
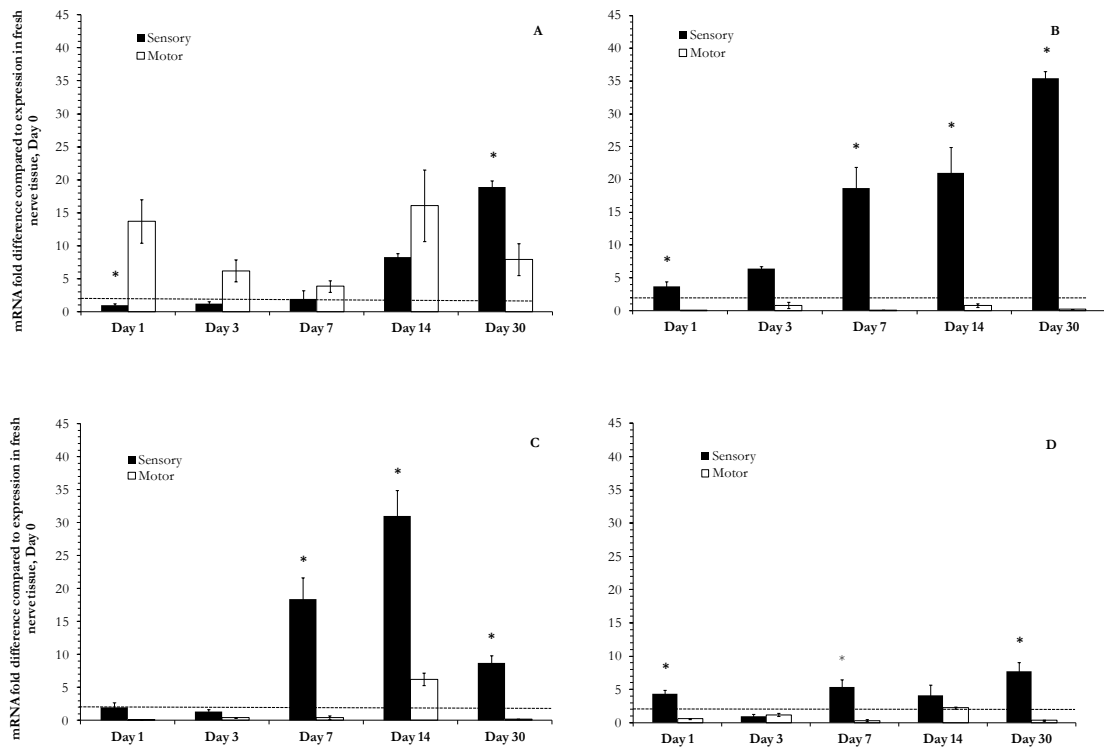
Figure 2.3. Gene expression in motor and sensory SCs over 30 days of *in vitro* culture by qRT-PCR. A) MBP B) NEFL C) PTN and D) PRKCi. The gene expression for each time point was normalized to β-actin, and then the fold difference versus fresh tissue (Day 0) was calculated. The error bars represent the standard deviation *(n =3)*. * denotes *p < 0.05* when compared to the gene expression in sensory SCs to motor SCs at that time point. *The dotted line at 2 is the threshold value for up regulation.*

2.5 Discussion

A number of studies have shown that the transplantation of SCs at the injury site improves the regeneration of peripheral nerves (Brenner, Lowe et al. 2005; Fox, Schwetye et al. 2005). However, SCs need to be expanded in culture to transplant a sufficient number of cells at the injury site. During expansion, it is necessary to understand the effects of expansion culture on SC phenotype. In this study, we used a rat femoral nerve model to identify a set of phenotypic markers (from literature and gene chips) to monitor the expression profiles while the SCs were expanded *in vitro*.

There are two types of SCs, myelinating and nonmyelinating, present in healthy nerves (Mirsky and Jessen 1996). Myelinating SCs associate with axons in a 1:1 ratio and aid in saltatory conduction. Alternatively, the nonmyelinating SCs associate with several non-myelinated axons that conduct signals with wave-like impulses. From previous studies, it has been shown that the cutaneous nerve and ventral root have ~20% and ~33% myelinated axons (MAx) respectively and ~80% and ~66% nonmyelinated axons (NMAx) respectively (Schmalbruch 1986; Castro, Negredo et al. 2008). These different ratios in the number of MAx versus NMAx suggests that there are different proportions of the myelinating and nonmyelinating SCs present in the nerves, which may have an effect on the differences in the expression profiles on a healthy nerve. However, in the denervated nerve, both myelinating and nonmyelinating SCs regress into a more immature phenotype (Mirsky and Jessen 1996; Jessen and Mirsky 2005). Similarly, when the SCs are harvested from the fresh tissue and expanded *in vitro* the SCs may be reverting to a similar immature phenotype due to the lack of cues from the environment (e.g. interaction with axons). Previous studies have shown that cutaneous nerve and ventral root SCs exhibit phenotypic differences, but also differentiate into their original phenotype during regeneration even after prolonged contact

with axons of the opposite phenotype (Hoke, Redett et al. 2006). The implication that SCs having different expression profiles, which may influence preferential motor regeneration (PMR), suggests that transplanting phenotype specific-SCs may be potentially a promising therapeutic strategy for PMR.

To monitor the expression level changes in the SCs harvested from the motor and sensory branches of the rat femoral nerve, phenotypic markers were chosen from the gene chip analysis and from previously identified markers in literature. Although the gene chip analysis revealed ~100 genes that were differentially regulated in the motor or sensory SCs, the phenotypic markers chosen for this study were chosen because of the implication in SC function in the PNS. Additionally, a few genes (VEGF, GDNF, NGF, BDNF, and PTN) were selected from previously published literature (Hoke, Redett et al. 2006). These five genes may not have been identified from the gene chip analysis due to the stringent criteria (Costigan, Befort et al. 2002) used to identify the phenotypic markers. However, the markers chosen from the gene chips and literature were validated using qRT-PCR to show that they express similar trends to literature and the gene chips (Figures 2.1 &2. 2).

As mentioned earlier, the genes we chose as sensory markers (MBP, NLGN1, BDNF and GDNF) were chosen based on the role each gene plays in sensory SC function (Song, Ichtchenko et al. 1999; Guennoun, Benmessahel et al. 2001; Robert, Guennoun et al. 2001; Jahn, Tenzer et al. 2009; Biswas, Reinhard et al. 2010). BDNF and GDNF were chosen from literature as markers of sensory SC expression because these growth factors are predominantly expressed in the cutaneous nerve derived from the rat femoral branch (Hoke et al. 2006). The motor markers chosen were PRKCi, NEFL, VEGF and PTN. PRKCi and NEFL have been implicated in aiding with motor SC signaling and myelination (Roberson, Toews et al. 1992; Tisdale 2000; Mamidipudi and Wooten 2002; Lariviere and Julien 2004).

The remaining two genes, VEGF and PTN, were chosen from literature because they are predominantly expressed in the ventral root after denervation (Hoke, Redett et al. 2006). Due to the involvement of these genes in SC function, these markers can be used to monitor the changes in phenotypic gene expression profiles as SCs are expanded *in vitro*.

The expression profiles for the cells harvested from the motor and sensory branches of the rat femoral nerve were monitored by evaluating the relative mRNA levels of each gene (Table 2.4) compared to the expression levels in fresh tissue. As hypothesized, the expression patterns of these genes were altered as the SCs were expanded *in vitro*. Of the nine genes evaluated, the genes that were dysregulated in culture were MBP, NEFL, PRKCi, and PTN.

A GeneGo network pathway analysis on the results obtained through the gene chip experiment revealed pathways that may contribute to the changes in gene expression observed during SC expansion. A subset of genes from the Sox family has previously been shown to contribute to the neuronal development. One particular gene, Sox6, controls the transcription of the MBP (Stolt, Schlierf et al. 2006). Because MBP, a sensory marker, was upregulated in the motor SCs during culture, Sox6 alone with other transcription factors, such as SP1, Oct6, and Krox20, may be either be over- or under-expressed in the cell, thus altering the expression of MBP (Kao, Wu et al. 2009). Additionally, interaction with the sensory neurons promotes the synthesis of intracellular progesterone in SCs and thus may control the expression of Krox20, which regulates the expression of MBP (Guennoun, Benmessahel et al. 2001; Robert, Guennoun et al. 2001). The absence of neurons in culture may affect the expression levels of MBP in the SCs.

NEFL interacts with the family of microtubule-associated proteins (MAP) (Frappier, Stetzkowski-Marden et al. 1991), which bind to tubulin subunits to support the assembly of

microtubules in neurons. SCs also express NEFL in the healthy nerve to support the axon as well as aid in efficient signal conduction (Roberson, Toews et al. 1992). The dysregulation of NEFL may be due to the fact that the SCs are no longer supporting axons in cell culture and thus altering the expression patterns in the sensory SCs when grown *in vitro* in the absence of neurons. PRKCi plays a role in axonal transport, microtubule dynamics as well as SC survival and intercellular signaling (Tisdale 2000; Mamidipudi and Wooten 2002; van Niel, Porto-Carreiro et al. 2006). In SCs, these interactions are necessary to regulate SC apoptosis as well as assisting with vesicle budding and exosome formation for extracellular signaling to other SCs and axons. After expansion in culture, the expression of PRKCi may be dysregulated because the SCs are not in contact with axons. The PTN gene is important during neural development and promotes neurite outgrowth, cell proliferation, and cell migration. In recent studies, PTN has been shown to aid in the guidance of axonal regeneration and muscle reinnervation after injury (Deuel, Zhang et al. 2002; Jin, Jianghai et al. 2009). The changes in the gene expression levels, especially at day 14, may be due to the fact that the SCs are missing the necessary cues from axons to keep the PTN expression levels similar to expression in fresh tissue.

Additionally, at different time points during the SC expansion culture, the SCs are treated with different chemicals and mitogens (Table 2.3) to eliminate fibroblasts and to promote the proliferation of the cells using mitogenic supplements. Previously, it is have been shown that de-differentiation of SCs may be linked to proliferation (Guertin, Zhang et al. 2005). However, recent studies have revealed that SC de-differentiation is independent of mitogenic signaling and also uncoupled to proliferation (Monje, Soto et al. 2010). De-differentiated SCs do not proliferate unless treated with mitogenic supplements, whereas differentiated post-mitotic SCs do not respond to mitogenic additions to the culture media.

Since differentiation of the SCs is dependent on high levels of intracellular cyclic adenosine monophosphate (cAMP), SCs in the present study may have de-differentiated in culture due to the decrease in intracellular cAMP levels and loss of signaling from the environment *in vivo* and thus proliferate in response to mitogenic additions to the media.

The de-differentiated SCs *in vitro* may be mimicking the de-differentiated state the SCs revert to after injury and may be awaiting cues from the environment to induce differentiation into its native phenotype. *In vivo,* this immature state promotes SC proliferation and secretion GFs to aid axonal regeneration (Jessen and Mirsky 2005). As the axons grow, cues in the environment, such as GFs (Chan, Cosgaya et al. 2001; Iwase, Jung et al. 2005), neurotransmitters (Vrbova, Mehra et al. 2009), or supporting cells such as fibroblasts (Parrinello, Napoli et al. 2010), may guide the SCs differentiation back into their native phenotype to support the regenerating axons. To understand the differentiation and maintenance of SC phenotypes, further studies need to be conducted to evaluate the effects of culturing SCs with different environmental cues.

In conclusion, phenotype specific genes are differentially expressed in the motor branch and sensory branches of the femoral nerve. Additionally, we observed that these gene expression patterns were disrupted when motor and sensory SCs were expanded in culture. These results suggest that although motor and sensory SCs have different phenotype, future studies need to be performed to identify the environmental cues that influence and maintain the SC phenotype in a healthy nerve. GFs as well as neurotransmitters and supporting cells may help maintain the SC phenotype *in vivo* as well as *in vitro.* Understanding the cues that guide differentiation or maintenance of SC phenotype may improve SC transplantation therapies for the improvement of motor or sensory specific regeneration across nerve gaps using ANGs after injury.

Chapter 3

Schwann Cells Seeded in Acellular Nerve Grafts Promotes Increased Growth Factor Expression When

3.1   Abstract

Peripheral nerve regeneration is dependent on trophic support after injury. Schwann cells (SCs) secrete growth factors that promote neuronal survival and guide axons during regeneration. However, to obtain a sufficient number of SCs for transplant, SCs must be expanded in culture, which causes dedifferentiation into immature SCs. The purpose of this study was to determine if injection of SCs into acellular nerve grafts (ANGs) enhances growth factor expression and promotes the differentiation of the SCs back into their native phenotype. Donor sciatic nerves were harvested and placed in University of Wisconsin solution to generate cold preserved (CP)-ANGs. SCs from sciatic nerve and both the motor and sensory branches of rat femoral nerve were expanded *in vitro* and injected into CP-ANGs to repair 14 mm sciatic nerve defects. At 14 days, motor or sensory-derived SCs increased expression of growth factors (glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF)) in CP-ANGs versus isografts. However, the SCs in the ANGs failed to express markers associated with their native (donor) phenotype (motor or sensory) 2 weeks after injection into CP-ANGs. The results suggest that inclusion of SCs from the rat femoral nerve in CP-ANGs promotes increased growth factor expression compared to the isograft, but the CP-ANGs do not provide sufficient cues to re-differentiate the SCs into their native phenotypes.

3.2 Introduction

Direct end-to-end anastomosis of a severed peripheral nerve provides the optimal clinical outcome following injury. In most clinical cases, primary nerve repair is not possible and a bridging component that allows for tension-free reconstruction must be used to achieve functional recovery(Siemionow and Brzezicki 2009). Nerve autografts, the standard for peripheral nerve reconstruction, are limited by lack of sufficient donor tissue and size mismatches with the injury site(Schmidt and Leach 2003; Burnett and Zager 2004). Fresh cadaveric allografts can function as well as nerve autografts(Bain, Mackinnon et al. 1989; Midha, Mackinnon et al. 1993; Nakao, Mackinnon et al. 1995; Strasberg, Hertl et al. 1996; Strasberg, Mackinnon et al. 1996), but require host immuno-suppression with its attendant morbidity. To circumvent these problems, investigators have sought alternatives in synthetic conduits and acellular nerve allografts, both of which are currently commercially available for clinical use in the United States.

Acellular nerve grafts (ANGs) are prepared using freeze thaw cycles(Zalewski and Gulati 1982; Gulati and Cole 1994), cold-preservation(Gulati and Cole 1994), or detergent(Hudson, Liu et al. 2004; Hudson, Zawko et al. 2004) treatment. These processing methods remove the antigenic cellular components, thus reducing the immunological response to ANGs. Compared with nerve conduits, ANGs support superior nerve regeneration, likely due to the intact endoneurial microstructure of extracellular matrix (ECM) proteins that guide regenerating axons(Whitlock, Tuffaha et al. 2009; Johnson, Newton et al. 2011; Moore, MacEwan et al. 2011). Surgical reconstruction of nerves using conduits has generally been limited to short gaps and sensory nerve defects due to their inferior regeneration potential compared to autografts(Mackinnon and Dellon 1990; Mackinnon and Dellon 1990; Meek and Coert 2002). Preclinical and early clinical studies on

commercially-available ANGs have shown regeneration across gap lengths up to 28 mm[(Whitlock, Tuffaha et al. 2009),(Karabekmez, Duymaz et al. 2009)]. However, the lack of SCs, which are critical to peripheral nerve regeneration, limits regeneration in ANGs and makes them inferior to autografts(Whitlock, Tuffaha et al. 2009; Moore, MacEwan et al. 2011)[,20-26].

In uninjured nerves, SCs myelinate axons and secrete both ECM molecules and growth factors to promote neuronal survival(Reynolds and Woolf 1993; Bunge 1994; Nagarajan, Le et al. 2002; Brenner, Lowe et al. 2005). After nerve injury, the SCs present at the injury are essential to regeneration. The SCs proliferate and align themselves in the remaining basal lamina to guide regenerating axons to their distal targets(Waller 1850; Burnett and Zager 2004). ECM molecules and soluble growth factors, such as nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), and brain derived neurotrophic factor (BDNF), are secreted by SCs to stimulate and guide axons from the proximal stump toward their target end-organ(Frostick, Yin et al. 1998; Chan, Cosgaya et al. 2001; Iwase, Jung et al. 2005). ANGs injected with SCs have been shown to support regeneration at levels similar to autografts(Guenard, Kleitman et al. 1992; Kim, Connolly et al. 1994; Levi, Guenard et al. 1994; Hu, Zhu et al. 2007; Aszmann, Korak et al. 2008).

To obtain a sufficient number of cells to transplant, SCs typically need to be expanded in culture. Previous research suggests that SCs exhibit a specific phenotype (i.e. motor or sensory) that may influence the regeneration of axons towards their correct target end-organ (muscle or sensory) (Brushart 1988; Brushart 1993; Madison, Archibald et al. 1996; Madison, Archibald et al. 1999; Hoke, Redett et al. 2006; Madison, Robinson et al. 2007; Madison, Sofroniew et al. 2009). Previously, we quantified phenotype-specific gene expression to confirm that the motor and sensory branches of the femoral nerve are a source of phenotype-specific SCs(Jesuraj, Nguyen et al. 2012). Based on these studies, we

hypothesized that the source of SCs (i.e., motor or sensory nerve) could enhance growth factor expression after transplantation into CP-ANGs. Rat SCs derived from the sciatic nerve and the motor and sensory branches of the femoral nerve were expanded *in vitro* and then transplanted (Figure 3.1) into CP-ANGs to determine if SC phenotype plays a role in upregulating growth factor expression at 2 weeks post-injury.
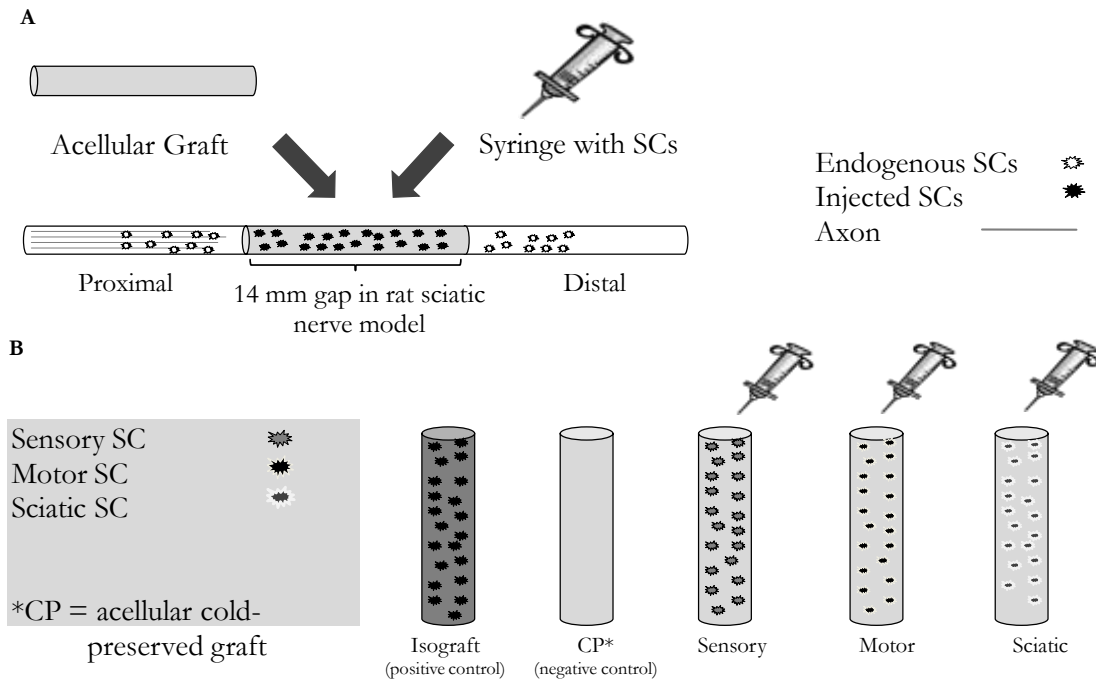
Figure 3.1. Experimental design for the transplantation study. The goal of the project was to transplant SCs into CP-ANGs to repair a 14 mm rat sciatic nerve gap defect to study the effects of CP-ANG ECM on growth factor and phenotypic marker expression patterns (A). Experimental groups included the isograft (positive control), the acellular CP-ANG (CP, negative control) and three SC-injected CP-ANG groups. SCs were derived from three different nerve sources: (i) rat femoral sensory branch (Sensory), (ii) rat femoral motor branch (Motor) and (iii) rat sciatic (mixed) nerve (Sciatic) (B).

3.3   Materials and Methods

3.3.1 Animals

Adult (225-250 g) male Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN) were maintained in a central housing facility. All animal procedures were approved and done in strict accordance with the guidelines set forth by the Animal Studies Committee of Washington University.

3.3.2 Experimental Design

Animals were randomized into five groups corresponding to the type of graft that was used to repair a 14 mm sciatic nerve gap. Additional animals were used as donors for nerve grafts or SCs. The first group (isograft) served as the positive control and received a 14 mm reversed isograft repair obtained from another Lewis donor. Three more groups received 14 mm CP-ANGs injected with 10^6 cells SCs derived and expanded from the sciatic nerve, femoral motor, and femoral sensory nerve branches respectively. The last group served as the negative control, receiving a 14 mm CP-ANG with no SCs (Figure 3.1 B).

3.3.3 Processing of Donor Nerve Grafts

Sciatic nerve segments from donor Lewis rats were immediately transferred into sterile six-well plates with 10 mL of a solution containing University of Wisconsin solution (Southard and Belzer 1995) (15 ml; NPBI International BV, Emmer Compascuum, The Netherlands), penicillin G (200,000 U/L), regular insulin (40 U/L), and dexamethasone (16

mg/L). The solution was changed weekly in a sterile hood for 7 weeks and stored at 4°C as described previously(Fox, Jaramillo et al. 2005).

3.3.4 Isolation and expansion of SCs

SC cultures were prepared as previously described (Raff, Abney et al. 1978; Brockes and Raff 1979; Pruss 1982). Briefly, the rat sciatic nerve and the sensory and motor branches of rat femoral nerve were harvested and placed in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Collagenase I (1%) (Fisher, Pittsburgh, PA) and trypsin (2.5%) (Invitrogen) were added to the fascicles and incubated for 30 min at 37°C. After centrifugation at 130 x g for 5 min, the pellet was washed with Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% antibiotic antimycotic (ABAM, Invitrogen). The cells were then seeded on 10 cm tissue culture dishes coated with poly-L-lysine (pLL) (Sigma-Aldrich). Tissue culture dishes were prepared by coating with 10 mL 0.01% pLL in sterile water and washing twice with sterile water. On day 2 of culture, 10 μM cytosine-beta-arabino furanoside hydrochloride (Sigma-Aldrich) was added to cultures along with the media containing DMEM, FBS, and ABAM. On day 6, the fibroblasts were complement-killed using an anti-Thy 1.1 antibody (1:40 dilution in media, Serotec, Raleigh, NC) and guinea pig complement (1:4 dilution in media, Sigma-Aldrich). On subsequent days the culture media was supplemented with 2 μM forskolin (Sigma-Aldrich), and 20 μg/mL pituitary extract (Biomedical Tech, Inc., Stoughton, MA).

3.3.5 Preparation of SCs for Injection

SCs were prepared for injection under the epineurium as previously described(Jesuraj, Santosa et al. 2011). Briefly, SCs were washed twice with Hanks' Balanced saline solution (Invitrogen) and incubated with 0.25% trypsin for 3 min at 37°C. After centrifugation for 5 min at 130 x g, the supernatant was removed, and the cells were resuspended at 10^6 cells/ 5μL in culture media containing DMEM supplemented with 10% FBS and 1% ABAM. A 10 nM Qtracker® (Invitrogen) solution was prepared for labeling, as recommended by the manufacturer. After mixing 200 μL of fresh media in 1.5 mL of prepared Qtracker® solution, 1x10^6 SCs were added and incubated at 37°C for 60 min to label the cells prior to transplantation. The resulting labeled cells were washed with media twice and concentrated as needed in culture media.

3.3.6 Donor Graft Harvest

Animals were anesthetized with a subcutaneous injection of ketamine (75 mg/kg, Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine (0.5 mg/kg, Dormitor®, Orion Corporation, Espoo, Finland). Under aseptic conditions, both hind limbs were prepared for incision. A 3 cm skin incision was made from the top of the femur towards the kneecap, and then the gluteal muscles were separated to expose the sciatic nerve. A 30-35 mm sciatic nerve segment was excised bilaterally and used for immediate isograft repair or cold-preservation treatment. The animals were subsequently euthanized with intracardiac injection of Euthasol® (150 mg/kg, Delmarva Laboratories, Des Moines, IA). All animal care was performed according to NIH guidelines and IUCAC approval.

3.3.7 Graft Implantation

With the experimental animals anesthetized, the right hind limb sciatic nerve was exposed, neurolysed, and sharply transected with micro-scissors 5 mm proximal to the trifurcation. The reversed 14 mm nerve graft was sutured to the proximal and distal stumps with one 10-0 nylon suture at each end and secured with fibrin sealant (Tisseel™, Baxter International Inc., Deerfield, IL). For groups that received SC treatments, injections were done after the CP-ANG was fastened to the proximal stump. With a 27-gauge Hamilton™ syringe (Hamilton Company, Reno, NV), a solution with 1×10^6 SCs/5 μL was injected longitudinally underneath the epineurium of the graft as previously described(Jesuraj, Santosa et al. 2011). To confirm adequate injection of the SCs, the labeled SCs were visualized in the CP-ANG with a fluorescence Olympus MVX10 dissecting microscope (Olympus Corporation, Japan) fitted with a cooled CCD digital camera (Hamamatsu ORCA-ER; Hamamatsu City, Japan) and analyzed with MetaMorph version 7.0 (Universal Imaging Corporation, PA). After wound irrigation, the muscles and skin were reapproximated with interrupted 6-0 Vicryl (Ethicon, Somerville, NJ) and 4-0 nylon sutures, respectively. Animals were recovered with a subcutaneous injection of atipamezole HCl (1mg/kg, Antisedan®, Orion Corporation) and placed on a warming pad post-operatively. Following surgery and post-operative care, animals were returned to a central housing facility and closely monitored for infection, distress, and other morbidities.

3.3.8 Graft Harvest

Animals used for qRT-PCR analysis were re-anesthetized 2 weeks after surgery. After the graft was identified and neurolysed, it was dissected at both suture sites to prevent any host nerve contamination(Aguayo, Charron et al. 1976; Aguayo, Epps et al. 1976), and the grafts were stored in RNA*later*™ (Ambion®, Austin, TX) for PCR analysis. Animals were euthanized with intracardiac injection of Euthasol® (150 mg/kg, Delmarva Laboratories, Des Moines, IA) immediately following harvest.

3.3.9 RNA Isolation

Total RNA was extracted from the explanted CP-ANGs 2 weeks after nerve repair using an acid phenol extraction (TRIzol Reagent, Invitrogen). The aqueous layer was collected, and the samples were purified using an RNeasy Mini Kit (Qiagen). The presence of RNA was assessed by electrophoresis using 2% agarose gels after running reverse transcriptase PCR with a β-actin primer. To verify that the mRNA extracted from the nerves met the quality standards for further experiments, mRNA concentration was determined using an absorbance ratio of A260/A280. The ratio threshold was at 1.8, which denotes a high purity of RNA in the sample(Wilfinger, Mackey et al. 1997). Since the majority of the cells in the nerve are SCs (~70%) (Salonen, Aho et al. 1988), the harvested RNA was assumed to be representative of SC RNA present in the explanted CP-ANGs after two weeks.

3.3.10 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

cDNA was synthesized from the isolated RNA using the Quantitect Reverse Transcription Kit (Qiagen). Using the Quantitect SYBR Green PCR Mastermix (Qiagen) in

combination with gene specific QuantiTect primer assays, qRT-PCR was performed using an Applied Biosystems 7000 Real-Time PCR thermocycler for genes chosen from literature and identified from previous experiments: vascular endothelial cell growth factor (VEGF), NGF, BDNF, pleiotrophin (PTN), GDNF, myelin basic protein (MBP), protein kinase C iota (PRKCi), neural cell adhesion molecule 1 (NCAM1), and neurofilament (NEFL)(Hoke, Redett et al. 2006; Jesuraj, Nguyen et al. 2012). The qRT-PCR was conducted using the following conditions: (1) 50°C for 2 min to eliminate any PCR products containing dUTP from carryover contamination; (2) 95°C for 15 min, to activate the polymerase; (3) 40 cycles of 95°C for 15 seconds to anneal, 55°C for 30 seconds to extend, and 72°C for 30 seconds to amplify with the fluorescent signal detected at 72°C(Gaumond, Tyropolis et al. 2006). Target genes were normalized to an internal control (β-actin) to account for variation in cDNA concentration between samples. No template was used as a negative control. The Quantitect primer assays are validated to have a PCR efficiency of 100%. The differences in gene expression levels between two different samples were calculated using the comparative delta crossover threshold (C_t) method(Livak and Schmittgen 2001)

3.3.11 Statistical Analysis

Statistical analyses were run using SigmaStat 3.0 (Systat Software, San Jose, CA). If ANOVA returned a statistically significant *p* value, a post-hoc Student-Newman-Keuls test was used to isolate significant differences between groups with correction for multiple comparisons. Significance was set at p<0.05 and all results are reported as mean ± standard deviation.

3.4   Results

qRT-PCR was used to study the effect of SC transplantation on growth factor expression in the acute phase after injury (Henderson, Camu et al. 1993; Li, Wu et al. 1995) with isografts and acellular CP-ANGs used as controls. NGF, GDNF and BDNF were chosen as representative growth factors because they promote neuronal survival and axon regeneration after peripheral nerve injury(Henderson, Camu et al. 1993; Li, Wu et al. 1995; Yan, Matheson et al. 1995; Xu, Yu et al. 2002; Lee, Yu et al. 2003; Wood, Moore et al. 2009). Two weeks after transplantation, gene expression in the nerve grafts was evaluated and compared to the expression levels in the negative control, acellular CP-ANGs. All growth factor expression levels (NGF, GDNF, and BDNF) were upregulated in the sensory and motor-derived SC groups compared to the acellular CP-ANG group (Figure 3.2). When compared to the isograft group, CP-ANGs injected with sensory and motor-derived SCs also showed increased expression of NGF (sensory ~4-fold increase, motor ~3-fold increase), GDNF (sensory ~10-fold, motor ~4-fold) and BDNF (sensory ~4-fold, motor ~ 3-fold) (Figure 3.2). The growth factor expression levels of the sciatic-derived SC group were similar to that of the isograft (Figure 2). The increased level of growth factor expression in the sensory and motor-derived SC groups compared to both the acellular CP-ANG and isograft groups demonstrates that transplantation of phenotype-specific SCs into CP-ANGs increases growth factor expression in this injury model after 2 weeks.
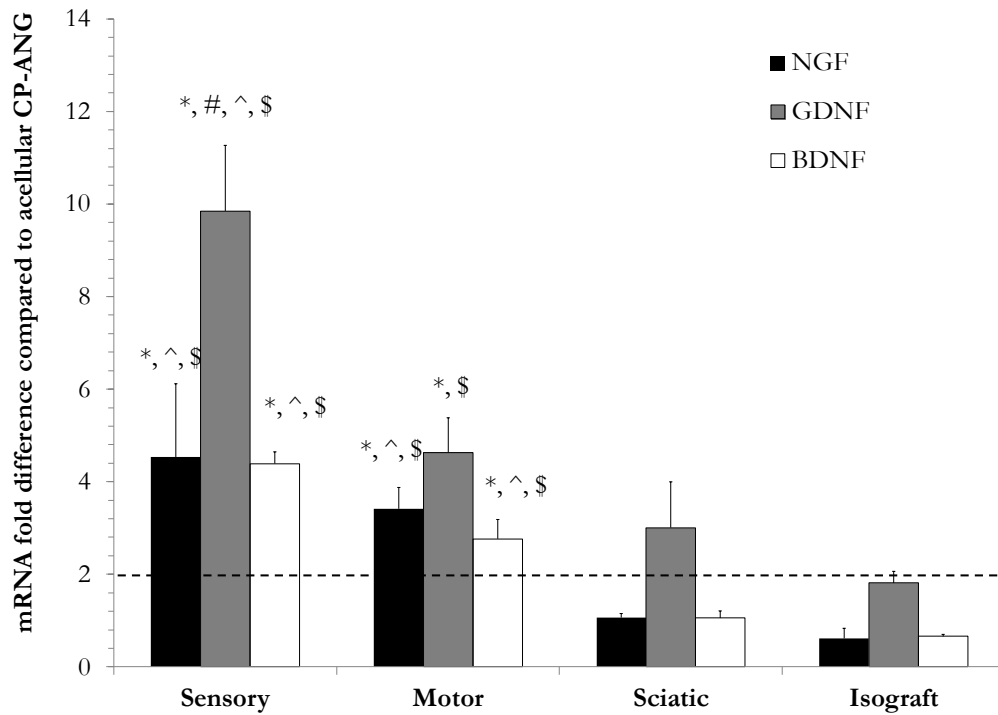
Figure 3.2. Sensory and motor-derived Schwann cells increase growth factor expression at 2 weeks. qRT-PCR was used to determine the gene expression level of each marker with the values normalized to β-actin. The groups injected with phenotype specific SCs (sensory and motor nerve-derived) showed greater expression of all growth factors examined compared to the isograft and acellular CP-ANG groups. The mRNA fold difference was calculated versus the acellular CP-ANG. ****** the dotted line at 2 is the threshold value for upregulation versus the CP group. Error bars represent the standard deviation (n = 3). ** denotes $p < 0.05$ when compared to isograft, ^ denotes $p < 0.05$ when compared to sciatic, # denotes $p < 0.05$ when compared to motor, $ denotes $p < 0.05$ when compared to acellular CP-ANG.*

Prior to transplantation, SCs were harvested and expanded in culture, which has been shown to affect the gene expression patterns of SCs(Jesuraj, Nguyen et al. 2012). To determine whether transplantation of SCs into CP-ANGs would promote differentiation of the SCs back into their native phenotypes, expression levels of phenotypic markers for sensory or motor-derived SCs (Table 3.1) (Jesuraj, Nguyen et al. 2012) were quantified. Of the phenotypic markers evaluated, only MBP and PRKCi showed significant changes in expression patterns compared to the other experimental groups. MBP is a previously identified marker of sensory-derived SCs(Jesuraj, Nguyen et al. 2012). However, in this study, MBP was upregulated 4-fold in the motor-derived SC group versus the sensory-derived SC group (Figure 3.3). Previously, PRKCi was reported to be a motor-derived SC marker(Jesuraj, Nguyen et al. 2012). Expression of PRKCi was 6-fold higher in the sensory SC group compared to motor SC group (Figure 3.3). In a previous study in our lab, we demonstrated that the expression of MBP and PRKCi were dysregulated in sensory and motor-derived SCs after 30 day expansion culture *in vitro*, compared to expression levels in fresh nerve tissue (Jesuraj, Nguyen et al. 2012). These results suggest that the phenotypic marker expression patterns of MBP and PRKCi remain dysregulated in the CP-ANGs 2 weeks post transplantation in this sciatic nerve injury model.

Table 3. 1: List of Genes used for qRT-PCR analysis 2 weeks post-transplantation

Gene	Gene Common Name	Upregulated in Motor, Sensory, or Similar
Vascular Endothelial Growth Factor	VEGF	Motor
Pleiotrophin	PTN	Motor
Protein Kinase C iota	PRKCi	Motor
Neurofilament	NEFL	Motor
Brain Derived Neurotrophic Factor	BDNF	Sensory
Glial Derived Neurotrophic Factor	GDNF	Sensory
Myelin Basic Protein	MBP	Sensory
Neural Cell Adhesion Molecule	NCAM1	Sensory
Nerve Growth Factor	NGF	Similar expression in both
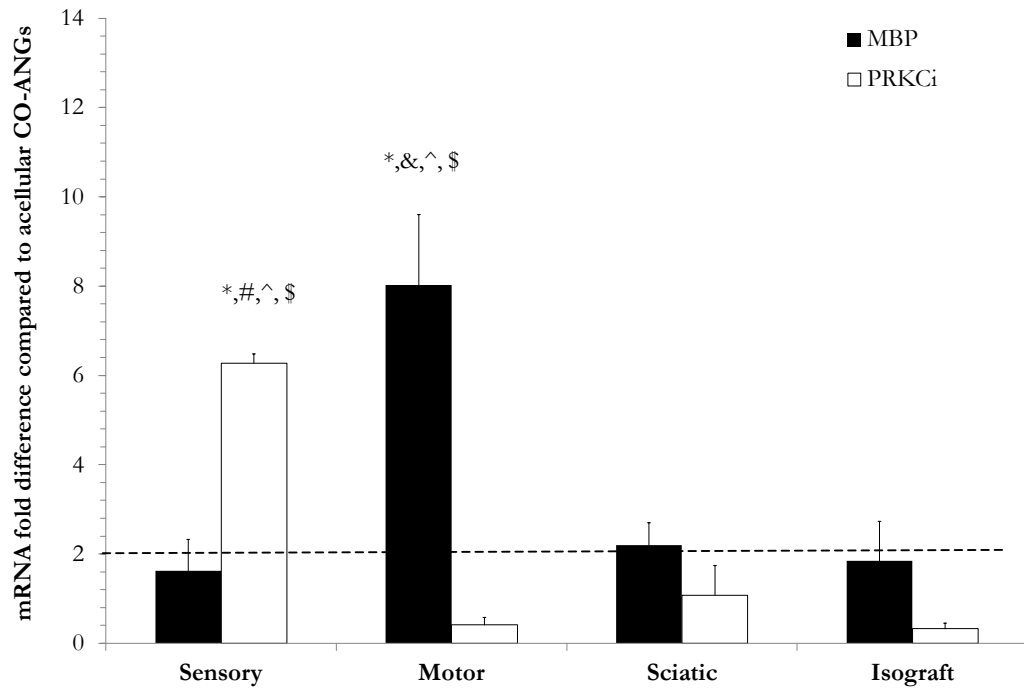
Figure 3.3. SC gene expression patterns remain dysregulated 2 weeks after transplantation in CP-ANGs. qRT-PCR was used to determine the gene expression of each marker with the values normalized to β-actin. MBP, a sensory marker, showed increased expression in the motor group when compared to all other groups. PRKCi, a motor marker, showed increased expression in the sensory group. The mRNA fold difference was calculated versus the acellular CP-ANG. **** the dotted line at 2 is the threshold value for upregulation versus the CP group. Error bars represent the standard deviation (n = 3). *denotes p< 0.05 when compared to isograft, & denotes p< 0 05 when compared to sensory, ^ denotes p< 0.05 when compared to sciatic, # denotes p< 0.05 when compared to motor, $ denotes p< 0.05 when compared to acellular CP-ANG.*

3.5   Discussion

Our study was designed to determine whether transplantation of SCs into CP-ANGs would increase growth factor expression and differentiation the SCs into their native phenoytpe in a 14 mm sciatic nerve injury model (a mixed nerve) and whether the source of SCs has an effect.   CP-ANGs were injected with rat SCs derived from three different sources, the sensory and motor branches of the femoral and the sciatic nerve, and nerve regeneration was evaluated using three metrics.   The effect of the SCs on growth factor and phenotypic marker expression was evaluated at 2 weeks using qRT-PCR.

We hypothesized that transplantation of motor-derived SCs into CP-ANGs would increase functional motor recovery through ANGs.   Pure motor SCs can be obtained from the ventral root(Hoke, Redett et al. 2006), but in a clinical setting the ventral root is difficult to access.   A more clinically relevant source of motor-derived SCs would be the long subscapular nerve, which innervates the latissimus dorsi muscle(Brown, Wickham et al. 2007).   In this study, motor-derived SCs were harvested from the motor branch of the femoral nerve, which we have previously shown to exhibit similar expression patterns of phenotypic markers as SCs derived from the ventral root(Hoke, Redett et al. 2006; Jesuraj, Nguyen et al. 2012).   To determine whether a population of mixed (motor and sensory) SCs would promote similar regeneration to motor and sensory SCs, SCs derived from the rat sciatic nerve were also used in this study.

Gene expression analysis showed that transplantation of sensory or motor-derived SCs into CP-ANGs increased expression levels of the growth factors NGF, BDNF, and GDNF in the grafts 2 weeks post transplantation compared to the acellular CP-ANG and isograft groups.   The increase in growth factor expression levels may be due to the expansion of SCs *in vitro* prior to transplantation.   Typically, SCs de-differentiate into an

immature state after injury, which promotes the upregulation of NGF, GDNF, and BDNF to guide the regenerating axons to their distal targets(Mirsky and Jessen 1996; Chan, Cosgaya et al. 2001; Iwase, Jung et al. 2005; Jessen and Mirsky 2005). Similarly, when the SCs are harvested from the fresh tissue and expanded *in vitro,* the SCs may revert to an immature phenotype and thus upregulate growth factor expression levels. Therefore, we hypothesized that inclusion of SCs into CP-ANGs would increase growth factor expression levels compared to CP-ANGs without SCs. Our results showed that expression levels of NGF, BDNF, and GDNF were higher in grafts with sensory-derived and motor-derived SCs compared to both the isograft and acellular CP-ANG groups. The differences in the growth factor expression levels compared to the isograft, which is sciatic-derived, may be due to the differential upregulation of the growth factors in different populations of SCs(Hoke, Redett et al. 2006; Jesuraj, Nguyen et al. 2012). Thus, the transplantation of sensory or motor-derived SCs into CP-ANGs increases levels of growth factor expression compared to both isografts and acellular CP-ANGs, which may help guide regenerating axons through CP-ANGs.

SCs derived from the sensory and motor branches of the rat femoral nerve have been shown to exhibit differential gene expression patterns(Jesuraj, Nguyen et al. 2012). However, as the SCs are expanded *in vitro*, the expression patterns of phenotypic markers, such as MBP (sensory marker) and PRKCi (motor marker), are dysregulated, possibly due to the lack of environmental cues *in vitro* (Jesuraj, Nguyen et al. 2012). Prior to transplantation, SCs need to be expanded in culture for approximately 30 days to obtain sufficient numbers of cells for transplantation, which means that the SCs used in this study are de-differentiated cells with dysregulated phenotypic expression patterns(Jesuraj, Nguyen et al. 2012). Therefore, we hypothesized that transplantation of these de-differentiated motor or sensory-

derived SCs into CP-ANGs would promote differentiation of the SCs and expression of their native phenotypic markers. Yet similar to *in vitro* findings (Jesuraj, Nguyen et al. 2012), the expression of MBP and PRKCi by SCs in the CP-ANGs remained dysregulated. These results suggest that the environment within a sciatic nerve-derived CP-ANG may not provide sufficient cues to re-differentiate SCs into their native phenotype within the two week period evaluated in this study. Other cues, such as phenotype specific axons regenerating through the CP-ANG or a later time point, may be required to observe differentiation of the SCs into their native phenotypes. Alternatively, if the CP-ANG was derived from primarily sensory or motor nerves, the graft might provide stronger cues to promote the expression of native phenotype-specific markers.

This study demonstrates the injection of SCs, regardless of source, into CP-ANGs promoted increased growth factor expression at 2 weeks compared to the isograft. Currently, SCs can be obtained from the transected nerve by removing a piece of nerve from the injured nerve stump. If the peripheral nerve source that the SCs are derived from does not have an effect on the growth factor expression, then SCs can be derived from the autologous nerve source that provides the least donor site morbidity. However, further work needs to be completed to understand which additional cues may be necessary to promote the differentiation of the SCs into their native phenotypes *in vivo*. Further studies should also assess the types of axons (motor or sensory) that are regenerating in response to transplantation of phenotype specific SCs. Understanding which factors may affect gene expression levels in SCs will allow for designing better cell transplantation therapies to promote peripheral nerve regeneration.

Chapter 4

Effects of Nerve Growth Factor, Glial-derived Neurotrophic Factor, and Acetylcholine on Schwann Cell Differentiation

4.1 Abstract

Schwann cells (SCs) secrete growth factors (GFs) and extracellular matrix (ECM) molecules that promote neuronal survival and help guide axons during regeneration. The addition of SCs to acellular nerve grafts is a promising strategy for enhancing peripheral nerve regeneration. To obtain a sufficient number of cells for transplant, SCs must generally be expanded *in vitro*. However, we and others have shown that after long-term *in vitro* expansion, SCs tend to revert to a de-differentiated state similar to the phenotype of SCs after injury. Because SCs express both the p75^{NTR} (nerve growth factor (NGF)) and GFRα-1 receptors (glial-derived neurotrophic factor (GDNF)), it is possible that NGF and GDNF present in the extracellular milieu may promote the differentiation of the SCs into a mature myelinating phenotype. In addition, small molecules, such as acetylcholine (Ach), are present in the extracellular milieu of motor nerves near the neuromuscular junction (NMJ) and may reinforce a motor SC phenotype. SCs express the muscarinic Ach receptors 1 through 4, and thus Ach may signal through these receptors. In this study, SCs harvested from the sensory and motor branches of rat femoral nerves were expanded *in vitro* and then cultured with different doses of NGF, GDNF, and Ach (motor-derived SCs only). The addition of NGF and GDNF to the media promoted the differentiation of both sensory and motor SCs back into their native phenotypes, as demonstrated by increases in S100 and phenotype-specific marker expression. Addition of Ach to the media of motor–derived SCs

also increased expression of S100, as well as motor SC markers. Because these GFs and Ach promote SC differentiation and phenotypic marker expression, these molecules can be incorporated into transplantation therapies to promote differentiation of transplanted immature cultured SCs for quicker myelination of the regenerating axons to improve functional recovery.

4.2   Introduction

Peripheral nerve injury due to a complete nerve transection results in a loss of sensory or motor nerve function. Ideally, the two severed ends of the nerves can be rejoined using a direct end-to-end coaptation. However, in larger nerve gap injuries, a direct coaptation can introduce unnecessary tension that may impede regeneration. To prevent tension while bridging a nerve defect, an autograft can provide SCs, ECM molecules and GFs to promote regeneration of axons across the nerve defect. Although the nerve autograft remains the clinical standard of care, this method has limitations including donor site morbidity, lack of sufficient donor tissue, and size mismatches at the injury site (Schmidt and Leach 2003; Burnett and Zager 2004). Currently, investigators are searching for alternative therapies to bridge nerve gaps following injury, such as acellular nerve grafts (ANGs).

ANGs have been used to support the growth of regenerating axons from the proximal nerve into the distal stump (Hare, Evans et al. 1993; Levi, Evans et al. 1994; Hare, Evans et al. 1995). However, the regenerative capacity of ANGs is still inferior to autografts because they lack SCs (Whitlock, Tuffaha et al. 2009). SCs secrete GFs and ECM that can promote neuronal survival and axonal regeneration after injury (Bunge 1993; Reynolds and Woolf 1993; Frostick, Yin et al. 1998; Nagarajan, Le et al. 2002; Brenner, Lowe et al. 2005). The addition of SCs to ANGs is a promising strategy for enhancing their regenerative capacity (Jesuraj et. *al*, unpublished data).

In addition to transplanting SCs into ANGs to promote regeneration, the transplantation of specific SC phenotypes (motor or sensory SCs) at the injury site to promote the regeneration of specific types of axons is an area of interest. When given equal

access to motor and sensory pathways, motor axons tend to regenerate down the motor pathway. This phenomenon, preferential motor reinnervation, may be influenced by trophic support from the end organs (with muscle greatly outweighing skin) or the SCs present within terminal nerve pathways (Brushart 1988; Brushart 1993; Madison, Archibald et al. 1996; Madison, Archibald et al. 1999; Madison, Robinson et al. 2007; Madison, Sofroniew et al. 2009). Previous studies have shown that sensory axons may also regenerate preferentially down sensory pathways (Hoke, Redett et al. 2006). After denervation, SCs de-differentiate into an immature state (Mirsky and Jessen 1996) and secrete GFs and ECM to enhance regeneration (Bunge, Bunge et al. 1986; Bunge 1993; Bunge 1994). Although SCs de-differentiate, they may retain a "phenotypic memory" that allows them to re-differentiate into their original phenotype during regeneration, as demonstrated by different expression profiles observed by motor and sensory SCs even after prolonged contact with axons of the opposite phenotype (Hoke, Redett et al. 2006).

To obtain a sufficient number of phenotype specific SCs for transplant, SCs need to be expanded from phenotype specific nerve sources. However, in a previous study, SCs derived from the motor (quadriceps) branch and the sensory (cutaneous) branch of the rat femoral nerve showed dysregulated phenotypic marker expression after long term *in vitro* expansion, possibly due to the lack of environmental cues(Jesuraj, Nguyen et al. 2012). *In vivo*, after nerve injury, the SCs present at the injury site de-differentiate into an immature phenotype to aid in the phagocytosis of the cellular debris and to promote the regeneration of axons (Waller 1850; Burnett and Zager 2004). As the axons sprout from the proximal end toward the distal end, the SCs secrete soluble growth factors, such as nerve NGF and GDNF (Frostick, Yin et al. 1998; Chan, Cosgaya et al. 2001; Iwase, Jung et al. 2005) to guide the axons to the correct target end-organ. As axons grow and reestablish contact with the

SCs, it has been hypothesized that interactions between the SCs and environmental cues (e.g. GFs and neurotransmitters) may be responsible in promoting the re-differentiation of the SCs into a mature phenotype to myelinate and support axon function (Mirsky and Jessen 1996; Jessen and Mirsky 2005).

In the regenerating nerve environment, the SCs are exposed to GFs, such as NGF and GDNF (Fu and Gordon 1997; Costigan, Befort et al. 2002). NGF and GDNF are GFs that have been shown to guide the regenerating axons across nerve defects (Otto, Unsicker et al. 1987; Nguyen, Parsadanian et al. 1998; Santos, Rodrigo et al. 1998; Barras, Pasche et al. 2002; Wang, Yang et al. 2002; Lee, Yu et al. 2003; Wood, Moore et al. 2009). Additionally, these GFs have also been shown to affect SC behavior, such as proliferation, migration, or differentiation. The binding of GFs to their respective surface receptors on the SCs (p75[NTR] for NGF and glycosylphosphatidylinositol (GPI)-anchored family receptor (GFR)α-1 for GDNF) may trigger downstream signaling to promote differentiation of the SCs into a mature phenotype (motor or sensory) (Chan, Cosgaya et al. 2001; Iwase, Jung et al. 2005).

Alternatively, the exposure to neurotransmitters may help to maintain and/or promote the differentiation SCs during remyelination of axons. Specifically, motor-derived SCs are exposed to Ach (Anderson and Stevens 1973) during regeneration of motor axons towards their target end-organ, muscle. The exposure of SCs to Ach may promote the expression of motor-specific SC markers(Jesuraj, Nguyen et al. 2012), which may further aid in the correct function of the regenerating axon.

However, to ensure that the transplanted cells will re-differentiate in their native phenotype as the axons regenerate, additional cues may need to be included into the transplantation therapies to treat nerve defects. To develop new SC transplantation therapies, it is important to understand which cues from the environment (e.g. NGF,

GDNF, and Ach) may promote differentiation into mature SCs. Therefore in this study, effects of NGF, GDNF, and Ach on SCs were evaluated the effect of NGF, GDNF and Ach concentration on SC maturation over 7 days. The extent of differentiation was evaluated using proliferation assays and quantitative real time polymerase chain reaction (qRT-PCR) to measure phenotypic marker expression.

4.3   Materials and Methods

4.3.1 SC Culture

SC cultures were prepared as previously described (Raff, Abney et al. 1978; Pruss 1982). Briefly, sensory and motor branches of the rat femoral nerve were harvested and placed in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Collagenase I (1%) (Fisher, Pittsburgh, PA) and trypsin (2.5%) (Invitrogen) were added to the fascicles and incubated for 30 min at 37°C. After centrifugation at 130 x g for 5 min, the pellet was washed with Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% antibiotic antimycotic (ABAM, Invitrogen). The cells were then seeded on 10 cm dishes coated with poly-L-lysine (pLL) (Sigma-Aldrich). Tissue culture plates were prepared by coating with 10 mL 0.01% pLL in sterile water and washing twice with sterile water. On day 2 of culture, 10 μM cytosine-beta-arabino furanoside hydrochloride (Sigma-Aldrich) was added to cultures along with the media containing DMEM, FBS, and ABAM. On day 6, fibroblasts were complement-killed using an anti-Thy 1.1 antibody (1:40 dilution in media, Serotec, Raleigh, NC) and rabbit complement (1:4 dilution in media, Sigma-Aldrich). On subsequent days the culture media was supplemented with 2 μM forskolin (Sigma-Aldrich) and 20 μg/mL pituitary extract (PE)

(Biomedical Tech, Inc., Stoughton, MA). Complement killing of fibroblasts was done as necessary, and the purity of the culture was assessed visually. After two passages, SCs were used for experiments.

4.3.2 Experimental design for dosage studies with NGF, GDNF, and Acetylcholine

Motor and sensory-derived SCs were seeded separately onto 48-well plates at 3000 cells/cm^2. Cells were cultured with unsupplemented media and supplemented media (0, 50, & 100 ng/mL NGF or GDNF for 7 days with media containing DMEM, ABAM, FBS and growth factor) changed every 2 days. SC pellets were then collected on days 3 & 7 for proliferation assay analysis or the RNA was collected from the SCs on days 3 and 7, and the RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). Samples were then stored at -80°C condition for further analysis.

For the Ach dosage study, motor-derived SCs, seeded onto 48-well plates at 3000 cells/cm^2, received 0, 0.01, or 0.1 mg/mL Ach in media (Sigma) for 7 days with media containing DMEM, ABAM, FBS and Ach changed every 2 days. SC pellets were then collected on days 3 & 7 for proliferation assay analysis or the RNA was collected from the SCs on days 3 and 7, and the RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). Samples were then stored at -80°C condition for further analysis.

To verify the effects of Ach on the SCs, gallamine, a blocker of both muscarinic and nicotinic Ach receptors, was used to block Ach receptors on the SC surface. Additional motor-derived SCs were seeded onto 48-well plates at 3000 cells/cm^2, and received 0 mM or 10 mM gallamine (Sigma) treatment for 2 hours, after which samples were dosed with 0, 0.01, 0.1, or 1 mg/mL Ach in media. RNA was collected from the SCs on day 7, and the

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). Samples were then stored at -80°C condition for further analysis.

4.3.3 Proliferation Assay

A CyQUANT ® cell proliferation assay was used to assess the number of cells present in culture after the different treatments, according to the manufacturer's protocol. Cells were lysed, and the number of cells was quantified using fluorescence using CyQUANT® GR dye, which strongly binds to nucleic acids.

4.3.4 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from the stored samples using the RNeasy mini kit (Qiagen). cDNA was synthesized from the isolated RNA using the QuantiTect® Reverse Transcription Kit (Qiagen). Using the QuantiTect® SYBR® Green PCR Mastermix (Qiagen) in combination with gene specific QuantiTect® primer assays, qRT-PCR was performed using an Applied Biosystems 7000 Real-Time PCR thermocycler. The genes studied included S100 (differentiated marker), nestin (undifferentiated marker), motor markers vascular endothelial cell growth factor (VEGF) and protein kinase C iota (PRKCi), and sensory markers brain derived neurotrophic factor (BDNF) and myelin basic protein (MBP). The primers for those mentioned genes were added to the cDNA for each sample present for the motor and sensory nerves. The qRT-PCR was conducted using the following conditions: (1) 50°C for 2 min (2) 95°C for 15 min, and (3) forty cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds (Gaumond, Tyropolis et al. 2006). Target genes were normalized to an internal control (β-actin) to account for the variation in cDNA concentration between samples, and appropriate negative control samples were

present (no template control). The QuantiTect® primer assays are validated to have a PCR efficiency of 100%. To estimate the mRNA concentrations, the differences in gene expression levels between two different samples were calculated using the comparative delta crossover threshold (C_t) method (Livak and Schmittgen 2001; Pfaffl 2001; Schmittgen and Livak 2008).

4.3.5 Statistical Analysis

Statistical analyses were performed using SigmaStat 3.0 (Systat Software, San Jose, CA), and all data were evaluated with one-way analysis of variance (ANOVA), followed by a Scheffe's F test for comparisons between groups when significance ($p < 0.05$) was present. All results are reported as mean ± standard deviation.

4.4   Results

4.4.1 Proliferation of SCs in Response to NGF and GDNF

Previous studies have shown that SCs expanded in culture revert to a proliferative, de-differentiated state in response to mitogenic media (Salzer, Williams et al. 1980; Morgan, Jessen et al. 1991; Monje, Soto et al. 2010). However, when SCs differentiate into mature cells, they transition into a post-mitotic state (Mirsky and Jessen 1996). To determine whether exogenous NGF or GDNF would slow the proliferation of sensory and motor-derived SCs, the number of cells present in culture was determined after 3 and 7 days of NGF or GDNF exposure.

The number of cells present after addition of NGF and GDNF to the media was determined at 3 and 7 days using a proliferation assay. Supplementation of both sensory and motor-derived SCs with 50 or 100 ng/mL of NGF or GDNF resulted in a lower number of cells than the unsupplemented control at both 3 and 7 days (Figure 1). The number of cells present after GDNF supplementation was similar to NGF for both types of SCs at each time point. The decrease in SC proliferation compared to unsupplemented media suggests that both NGF and GDNF may be driving the SCs into a post-mitotic state.
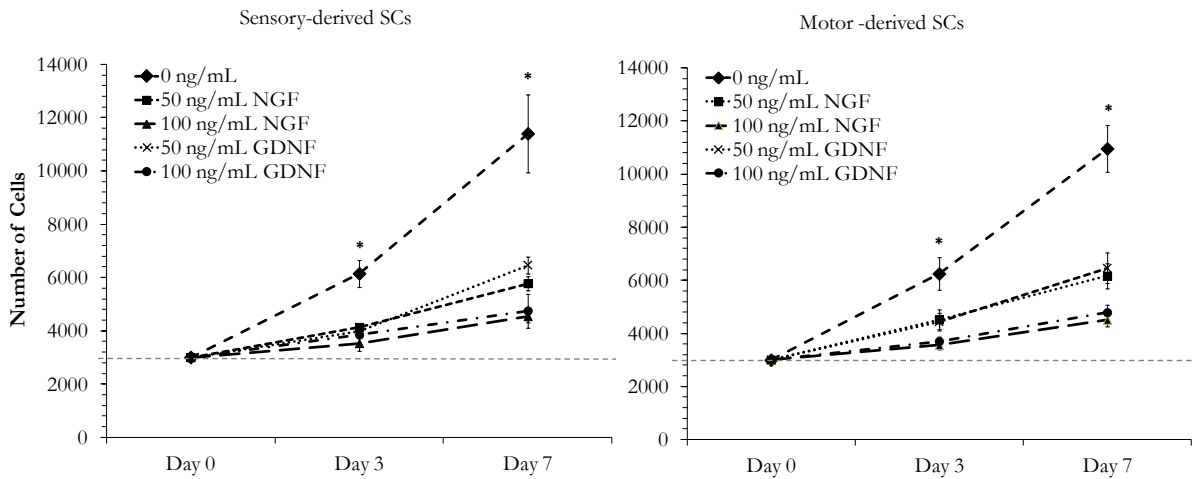
Figure 4.1. Addition of GFs to the SC media promotes decreased proliferation of SCs. The proliferation of sensory or motor-derived SCs was monitored over 7 days. The SCs were supplemented with NGF or GDNF at 0, 50 or 100 ng/mL. Error bars represent standard deviation (n=6). Dotted line represents initial seeding density. * denotes $p < 0.05$ compared to the same time point in the GF treated groups.

4.4.2 Differentiation of SCs in response to NGF and GDNF

To verify that SCs with GFs in the media were differentiating, qRT-PCR was performed on the cell lysates collected at 3 and 7 days for SCs with and without NGF or GDNF in the culture media. The differentiation of the SCs was evaluated using S100, which is a marker of SC maturity (Brockes, Fields et al. 1979; Raff, Brockes et al. 1979). Increased expression of S100 in SCs cultured with NGF or GDNF suggested differentiation towards a more mature phenotype. Nestin, which is a marker of undifferentiated SCs (Brockes, Fields et al. 1979; Raff, Brockes et al. 1979), was used to determine the presence of de-differentiated SCs after treatment with GFs. For both sensory and motor-derived SCs, the addition of NGF (Figure 4.2) or GDNF (Figure 4.3) to the media increased the expression of S100 and decreased the expression of nestin at 3 and 7 days in a dose dependent manner (Figures 4.2 & 4.3). This expression pattern suggests that the GFs may trigger downstream signaling within the cell to differentiate the SCs into a more mature phenotype (Hirata, Hibasami et al. 2001; Nicol and Vasko 2007).

The increased expression of S100 suggested that GFs may also promote native phenotypic marker expression patterns in sensory and motor-derived SCs. In previous studies, markers for both sensory and motor-derived markers were identified, and it was also shown that these phenotype-specific markers are dysregulated when SCs are expanded in culture (Hoke, Redett et al. 2006; Jesuraj, Nguyen et al. 2012). The addition of NGF to sensory or motor-derived cultures promoted the increased expression of BDNF and MBP (sensory SC markers) in sensory but not motor-derived SCs in a dose-dependent manner (Figure 4.4A). Expression of motor SC markers (VEGF and PRKCi) was not stimulated by NGF in either type of SC (Figure 4.4B). Culturing SCs with GDNF promoted the increased expression of sensory markers (BDNF and MBP) in sensory-derived SCs (Figure 4.5A), and

motor markers (VEGF and PRKCi) in motor-derived SCs (Figure 4.5B). The increased

expression of sensory and motor SC marker with GF treatment suggests that NGF and

GDNF promote the re-differentiation of the SCs into their native phenotypes *in vitro.*
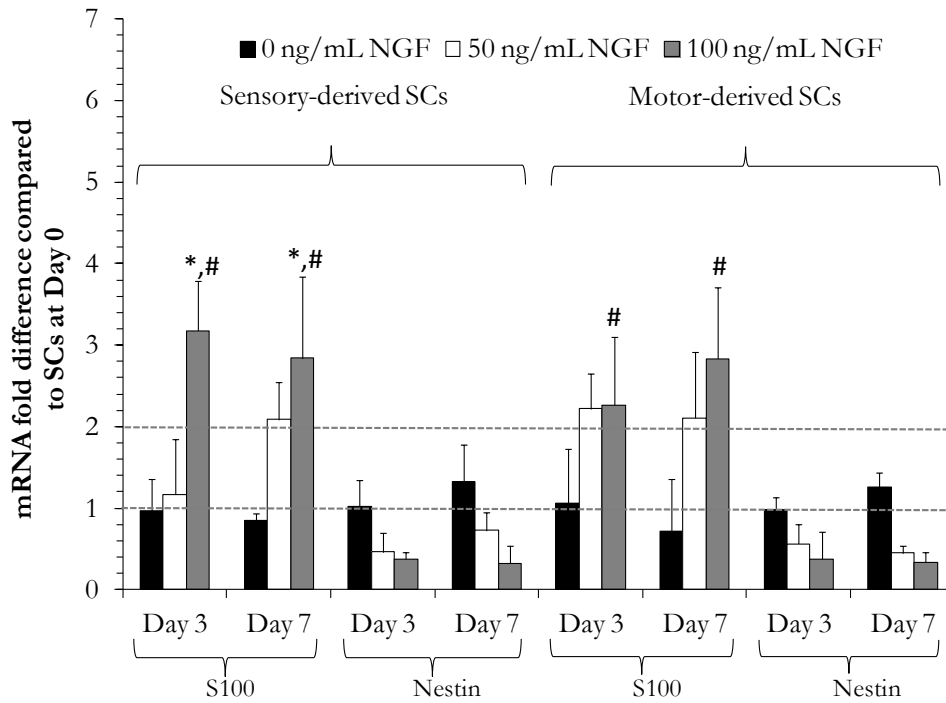
Figure 4.2. Addition of NGF to the media promotes increased expression of S100 and decreased expression of nestin. The expression of S100 and nestin was monitored over 7 days. qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The fold difference in gene expression for sensory and motor-derived SCs grown with and without NGF was compared to day 0. Error bars represent the standard deviation (n=3). ** the dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes p < 0.05 vs. 50 ng/mL at the same time point, # denotes p < 0.05 vs. 0 ng/mL at the same time point.

Figure 4.3. Addition of GDNF to the media promotes increased expression of S100 and decreased expression of nestin. The expression of S100 and nestin was monitored over 7 days. qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The fold difference in gene expression for sensory and motor-derived SCs grown with and without GDNF was compared to day 0. Error bars represent the standard deviation (n=3). ** the dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes $p < 0.05$ vs. 50 ng/mL at the same time point, # denotes $p < 0.05$ vs. 0 ng/mL at the same time point.

Figure 4.4. NGF in the SC media promotes increased expression of sensory markers in sensory-derived SCs. The expression patterns of BDNF and MBP (sensory markers) (A) and VEGF and PRKCi (motor markers) (B) was monitored over 7 days after addition of NGF to the cell culture. The fold difference of gene expression for SCs grown with and without NGF was compared to day 0 (passaged SCs). qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The mRNA fold difference was compared to the day 0. Error bars represent the standard deviation (n=3). The dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes p < 0.05 vs. 50 ng/mL of GF at the same time point. # denotes p < 0.05 vs. 0 ng/mL of GF at the same time point.

Sensory Markers
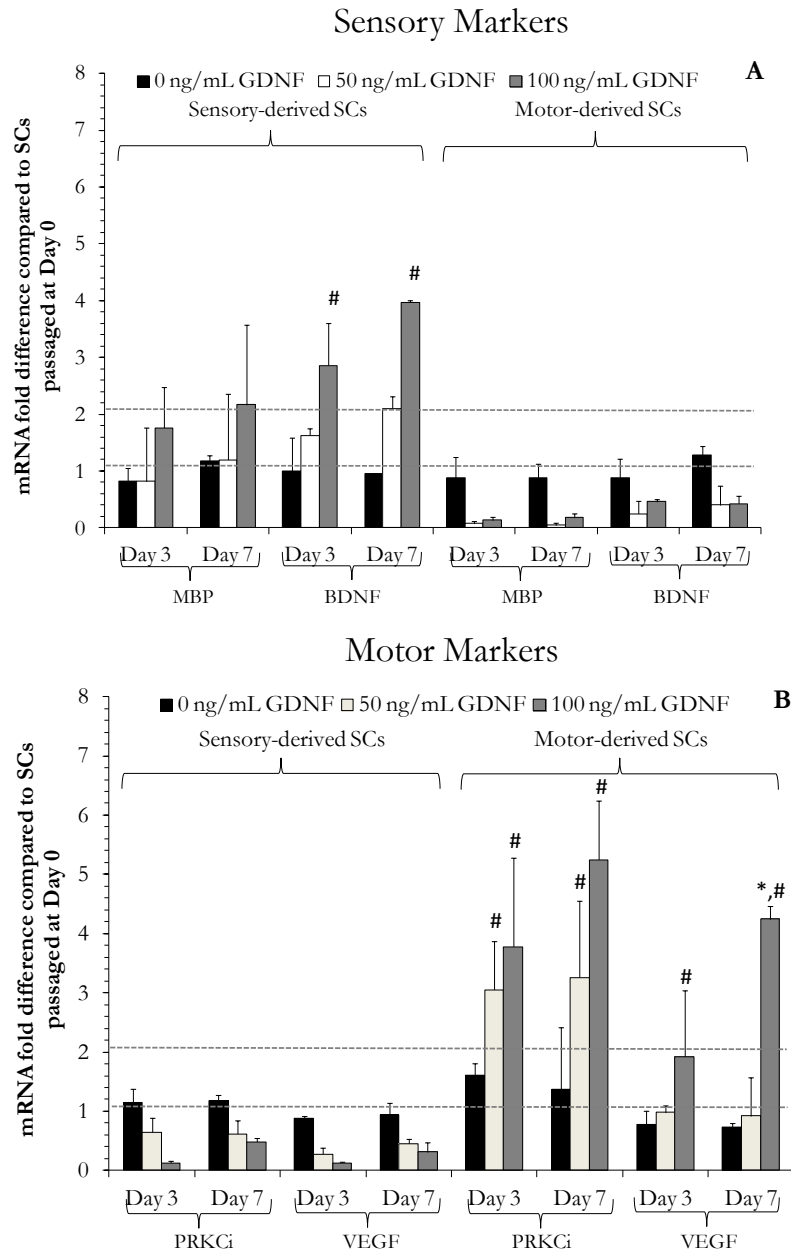
Motor Markers

Figure 4.5. GDNF increased the expression of sensory markers in sensory-derived SCs and motor markers in motor-derived SCs. The expression patterns of BDNF and MBP (sensory markers) (A) and VEGF and PRKCi (motor markers) (B) was monitored over 7 days after addition of GDNF to the cell culture. The fold difference of gene expression for SCs grown with and without GDNF was compared to day 0 (passaged SCs). qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The mRNA fold difference was compared to the day 0. Error bars represent the standard deviation (n=3). The dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes $p < 0.05$ vs. 50 ng/mL of GF at the same time point. # denotes $p < 0.05$ vs. 0 ng/mL of GF at the same time point.
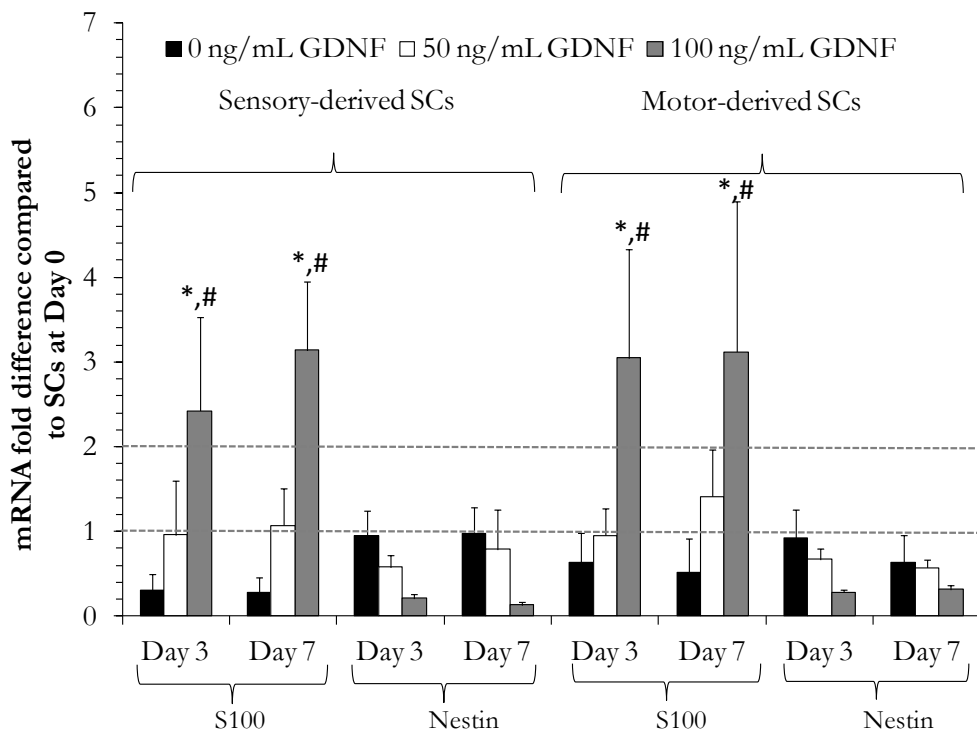
4.4.3 Proliferation of SCs in Response to Ach

Ach may also have an effect on SC differentiation, particularly for motor-derived SCs. Ach was added to SC culture media for 7 days and then a proliferation assay was performed to determine the effect on SC proliferation. The number of cells present after 7 days in the group treated with 0.1 mg/mL of Ach was lower than the groups treated with no Ach or with 0.01 mg/mL Ach. The number of SCs present in the 0 mg/mL and the 0.01 mg/mL groups after 7 days was approximately 3 to 3.5 times greater than the number of cells seeded at Day 0. The group treated with 0.1 mg/mL Ach only had about 2 times more cells (Figure 4.6). Because the SCs in the 0.1 mg/mL Ach group exhibited less proliferation than the 0 mg/mL and 0.01 mg/mL suggests that the Ach may be either affecting the proliferation of the SCs or promoting differentiation of the SCs.
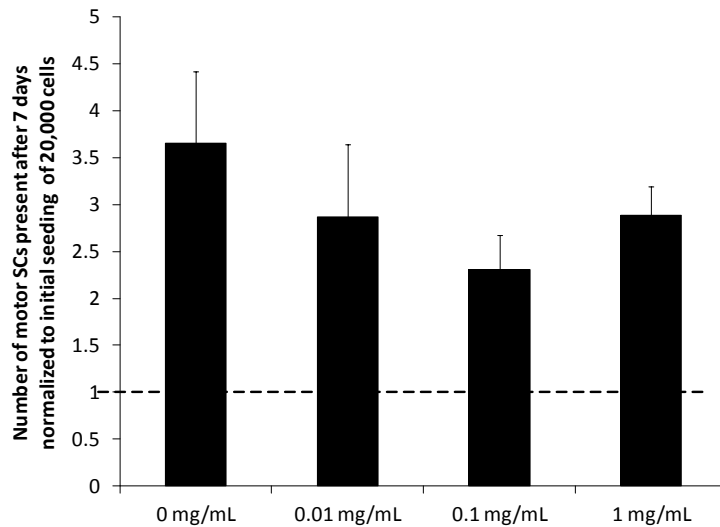
Figure 4.6. Addition of Ach to motor-derived SCs affects the proliferation of the SCs over 7 days. The proliferation of motor-derived SCs was monitored over 7 days. The SCs were supplemented with 0.01, 0.1, and 1 mg/mL of Ach. Error bars represent standard deviation (n=6). Dotted line represents initial cell number of 20,000 cells on day 0. * denotes $p < 0.05$ compared to the 0 mg/mL group, # denoted $p < 0.05$ compared to the 0.01 mg/mL group.

4.4.4 Differentiation of SCs in response to Ach

The trend present in the proliferation assay results suggested that the addition of Ach to the cultures at certain concentrations may promote SC differentiation, which may result in increased motor SC marker expression. The expression levels of S100 and nestin were evaluated using qRT-PCR at 3 and 7 days. The addition of Ach to motor-derived SCs promoted a biphasic response in the expression of S100 and nestin. The high (1.0 mg/mL) and low (0.01 mg/mL) doses of Ach resulted in lower expression of S100 compared to the 0.1 mg/mL dose (Figure 4.7). This biphasic response was also observed in the expression levels of motor markers. The addition of 0.1 mg/mL of Ach to motor-derived SCs promoted increased expression of motor SC markers (VEGF and PRKCi) compared to the other 2 doses of Ach at days 3 and 7 (Figure 4.8B). The expression patterns of sensory SC markers (BDNF and MBP) were similar to the expression level of SCs at day 0 (Figure 4.8A), suggesting that Ach did not promote expression of sensory markers in motor-derived SCs. The increased expression of differentiation and motor SC markers suggests that Ach may be playing a role in reinforcing and promoting the mature motor SC phenotype *in vitro*. To ensure that the observed effects were indeed due to the interaction of Ach with its receptors on the SCs, the Ach receptors were blocked with gallamine, a nicotinic and muscarinic receptor blocker, and the expression levels of motor SC markers were determined. Inhibition of Ach receptors with gallamine blocked the expression of motor SC markers in motor-derived SCs exposed to Ach even at 7 days (Figure 4.9). The combination of these results suggests that addition of Ach to motor-derived SCs promotes differentiation of SCs into a mature phenotype expressing motor SC markers.
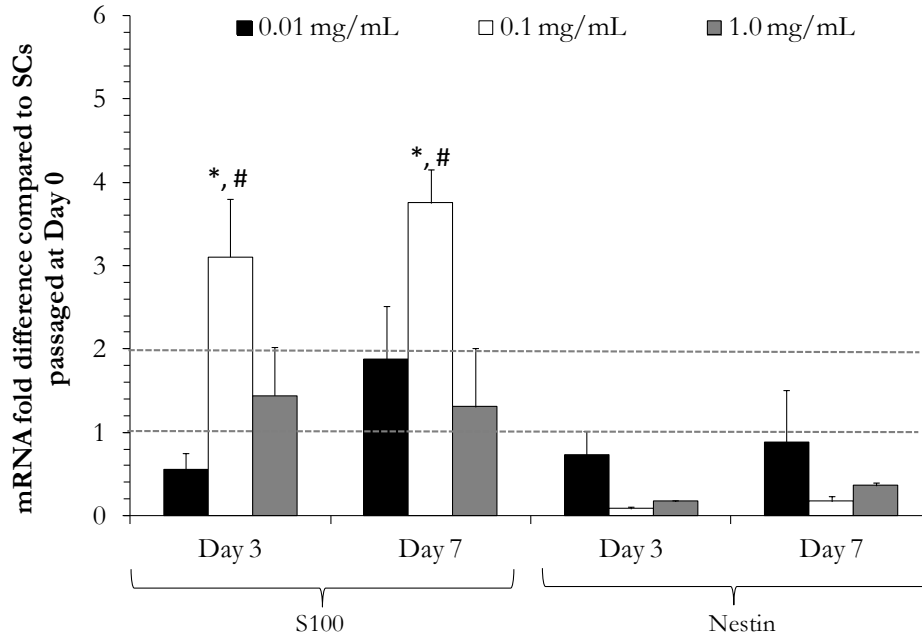
Figure 4.7. Addition of Ach to the media promotes increased expression of S100 and decreased expression of nestin. The expression of S100 and nestin was monitored over 7 days. qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The fold difference in gene expression for the motor -derived SCs grown with and without at different doses of Ach was compared to day 0. Error bars represent the standard deviation (n=3). ** the dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes p < 0.05 vs. 0.01 mg/mL at the same time point, # denotes p < 0.05 vs. 1.0 mg/mL at the same time point.
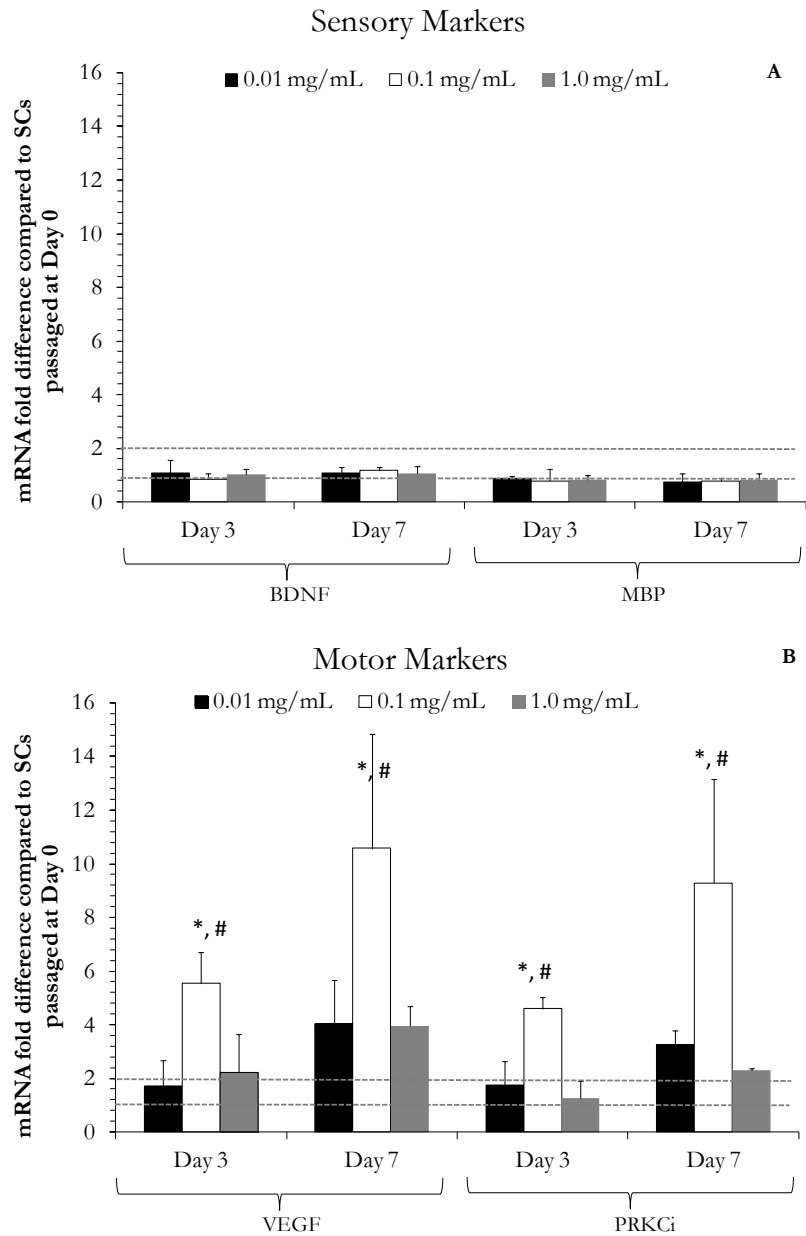
Figure 4.8. Ach in media promotes increased expression of motor markers in motor-derived SCs. The expression patterns of BDNF and MBP (sensory markers) (A) and VEGF and PRKCi (motor markers) (B) was monitored over 7 days. The fold difference of gene expression for SCs grown with and without Ach was compared to day 0. qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The mRNA fold difference was compared to the day 0. Error bars represent the standard deviation (n=3). The dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0. * denotes $p < 0.05$ vs. 0.01 mg/mL at the same time point, # denotes $p < 0.05$ vs. 1.0 mg/mL at the same time point.

Figure 4.9. Blocking of Ach receptors on SC surface prevents expression of motor markers in motor-derived SCs. The Ach receptors were blocked with 10 mM gallamine, different doses of Ach were added to the cell culture media, and the expression pattern of VEGF and PRKCi was evaluated over 7 days. The fold difference of gene expression for SCs grown with and without Ach was compared to day 0. qRT-PCR was used to determine the gene expression of each marker and the values were normalized to β-actin. The mRNA fold difference was compared to the day 0. Error bars represent the standard deviation (n=3). The dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to day 0.

4.5   Discussion

Understanding the environmental cues that affect the differentiation of immature SCs that have been expanded in culture into mature SCs is important in the designing SC-based therapies for peripheral nerve injury. Cues that may decrease SCs proliferation and promote differentiation include GFs or neurotransmitters. GFs, such as NGF and GDNF, are known to promote the regeneration of the axons, as well promote the migration and differentiation of SCs *in vivo*. Additionally, motor-derived SCs in an uninjured motor nerve are exposed to neurotransmitters, such as Ach, at the NMJ, which may help reinforce the

motor SC phenotype especially during nerve regeneration. Therefore, this study was designed to evaluate the effects of NGF, GDNF, and Ach on the proliferation of SCs and the expression patterns of the differentiation markers (S100 – mature, nestin – undifferentiated) and phenotypic markers (sensory SC – BDNF & MBP and motor SC – VEGF & PRKCi).

To transplant a sufficient number of SCs, the SCs must first be expanded *in vitro*. However, the long term expansion of SCs in culture promotes their de-differentiation and dysregulation of phenotypic marker expression patterns(Jesuraj, Nguyen et al. 2012). Although it has been shown that transplanting immature cultured SCs at the injury site is beneficial and promotes regeneration similar to the isograft (Jesuraj, unpublished data), this approach needs to be supplemented with additional cues to obtain better regeneration and functional recovery. More importantly, SCs may provide the trophic support necessary to guide regenerating motor or sensory axons to their correct target end organs. Without signaling from the environment, the SCs de-differentiate into an immature state *in vitro*, similar to the state after nerve injury. Thus it is very important to assess different environmental cues that signal the immature cultured motor or sensory-derived SCs to differentiate into their appropriate native phenotype.

Previously, it has been shown that there is a link between the de-differentiation and proliferation of SCs (Guertin, Zhang et al. 2005). However, recent studies have revealed that SC de-differentiation is independent of mitogenic signaling and also uncoupled to proliferation (Monje, Soto et al. 2010). De-differentiated SCs do not proliferate unless treated with mitogenic supplements, whereas differentiated post-mitotic SCs do not respond to mitogenic supplements (Salzer, Williams et al. 1980; Morgan, Jessen et al. 1991; Monje, Soto et al. 2010). Therefore, it has been hypothesized that the addition of NGF, GDNF,

and Ach to the media will not promote SC proliferation, but rather will promote the differentiation of SCs towards a mature phenotype that expresses sensory or motor markers. When sensory and motor-derived SCs were treated with different doses of NGF and GDNF, cell proliferation was decreased over 7 days, which indicates that the SCs may be differentiating into a mature phenotype. The addition of Ach to motor-derived SCs did not reduce the proliferation, but did show a trend of lower number of cells compared to the no supplement group after 7 days. This trend suggests that the Ach may have an effect on motor-derived SC differentiation. Evaluating the gene expression levels of differentiation and phenotypic markers further validated the effects of GFs and Ach on SC proliferation. The different downstream signaling pathways of NGF, GDNF and Ach may provide an explanation as to why these factors have different effects on the SCs.

Similar to sensory axons, SCs also express the p75^{NTR} NGF receptor (Hirata, Hibasami et al. 2001; Tomita, Kubo et al. 2007), which when stimulated regulates migration, apoptosis and SC differentiation through multiple signaling pathways (Bentley and Lee 2000; Segal 2003; Nicol and Vasko 2007). In this study, the addition of NGF to the media slowed the proliferation of SCs, suggesting that the NGF either activated apoptotic pathways allowing only some cells to proliferate, or it promoted differentiation. Gene expression analysis showed that NGF addition to the media promoted differentiation into mature SCs, as demonstrated by the increase in S100 expression in both sensory and motor-derived SCs. Interestingly, NGF only promoted increased sensory marker expression in sensory-derived SCs, which reinforces the concept that NGF is primarily a sensory nerve supporting GF and therefore only affects phenotypic marker expression in sensory-derived SCs.

Similarly, addition of GDNF to the culture media promoted the differentiation of both sensory and motor-derived SCs, as seen by increased expression of S100 and decreased

expression of nestin. GDNF also increased the expression of phenotype-specific marker in both types of SCs: GDNF stimulated increased expression of sensory markers in sensory-derived SCs and motor markers in motor-derived SCs. Exogenous GDNF has been shown to promote SC proliferation and myelination of normally unmyelinated small axons (Hoke, Ho et al. 2003). Mechanistically, GDNF has been shown to activate pathways in SCs, through the GFRα-1 surface receptor (Naveilhan, ElShamy et al. 1997), implicated in cell migration, proliferation, differentiation and GF production (Morgan, Jessen et al. 1991; Lang, Gesbert et al. 1996; Kim, DeClue et al. 1997; Klemke, Cai et al. 1997; Grimm, Holinski-Feder et al. 1998; Verity, Wyatt et al. 1998; Meintanis, Thomaidou et al. 2001; Ellerbroek, Wennerberg et al. 2003; Kinameri and Matsuoka 2003; Iwase, Jung et al. 2005). In this study, the addition of GDNF to the media slowed the proliferation of the SCs and promoted the expression of differentiation markers. The decrease in the proliferation of SCs suggests that exposure to GDNF promoted differentiation and a post-mitotic state (Mirsky and Jessen 1996). The combined results of stimulation of stimulating the $p75^{NTR}$ with NGF and GFRα-1 with GDNF demonstrate that with correct cues SC memory of phenotype can be restored *in vitro.*

SCs have also been shown to express cell surface receptors for Ach, which is present in the extracellular milieu of motor nerves. SCs express muscarinic receptors M1 through M4 for Ach, with M2 expressed at the highest level (Loreti, Vilaro et al. 2006). Previous work has established that Ach does not affect the viability of SCs (Salzer and Bunge 1980). In a recent *in vivo* study, it was shown that blocking of Ach receptors on SCs prevented the regeneration of motor axons into the distal nerve stump, suggesting that interaction of Ach with its receptor is necessary for motor nerve regeneration (Vrbova, Mehra et al. 2009). Therefore, in the present study, it was hypothesized that stimulation of Ach receptors would

promote the expression of motor markers in motor-derived SCs. Addition of Ach at different doses increased the expression levels of the differentiation marker S100 and motor SC markers in a biphasic manner. This biphasic response may be explained by the different types of muscarinic receptors on the SC surface and their role in modulating intracellular signaling and due to elevation in cyclic adenosine monophosphate levels, which may lead to SC differentiation (Felder 1995; Meintanis, Thomaidou et al. 2001; Monje, Soto et al. 2010). Furthermore, blocking Ach receptors on motor-derived SCs with gallamine did not promote the expression of the motor markers in the SCs, which once again reinforces the idea that interaction of Ach with SC muscarinic receptors is necessary for motor marker expression.

Although NGF, GDNF, and Ach have been shown to have positive effects on SCs in this study, further work needs to be done to incorporate SCs and these cues into therapeutic devices or grafts. Using a combinatorial strategy, NGF and GDNF can be incorporated into a delivery system within a scaffold such as fibrin (Sakiyama-Elbert and Hubbell 2000; Sakiyama-Elbert and Hubbell 2000), in which SCs could be transplanted at the injury site as well as be exposed to GFs to promote differentiation into specific SC phenotypes. Prior to transplanting SCs with GFs, additional studies need to be done to verify that the SCs still differentiate into a sensory or motor phenotype while exposed to NGF or GDNF within a scaffold.

Although Ach does stimulate the differentiation and the expression of motor SC markers in motor-derived SCs, using Ach to treat peripheral nerve injuries is not a clinically viable option. Ach is a neurotransmitter that is known to not only excite neurons, but it can also act as an inhibitory molecule and slow the heart rate. If Ach were released into the bloodstream during regeneration, it may be detrimental to cardiac function (DiFrancesco,

90

Ducouret et al. 1989). Therefore an alternative method to stimulate the Ach receptors could be explored.

Understanding how SC gene expression changes during de-differentiation and following transplantation could enable motor or sensory specific nerve regeneration using acellular nerve grafts seeded with phenotype specific SCs. In this study, we showed that NGF, GDNF, and Ach can be used to stimulate receptors on sensory and motor-derived SCs to promote differentiation into sensory or motor marker expressing SCs *in vitro*. Using this data along with previously established delivery systems, these cues can be incorporated into designing combinatorial therapies with SCs. Therefore, designing therapies and molecules to activate the myelination program in response to surface receptor stimulation in SCs will contribute to faster myelination of the regenerating axons, and thus promote faster and more robust regeneration of the peripheral nerves.

Chapter 5

Summary and Future Directions

5.1   Summary of Findings

This thesis work demonstrated that GFs and Ach present in the SC environment may contribute to the differentiation and phenotype-specific marker expression of SC. Demonstrated through three studies, the main goals of the studies were (1) to identify phenotypic markers of sensory-derived and motor-derived SCs from the rat femoral nerve and to monitor their gene expression patterns as SCs were expanded *in vitro,* (2) to transplant the SCs from different nerve sources (rat sciatic nerve, and the motor and sensory nerve branches of the rat femoral nerve) into ANGs to determine their effects on expression of GFs and phenotypic markers, and (3) to study the effects of GFs and Ach on the expression of differentiation and phenotypic markers *in vitro.*

The main goal of the first study was to identify a set of phenotypic markers that can be used to study gene expression patterns of sensory-derived and motor-derived SCs as they are expanded in culture. RNA was extracted from the sensory and motor branches of the rat femoral nerve. Using qRT-PCR and gene chips, phenotypic markers for both sensory and motor phenotypes were identified (from gene chip analysis and the literature) and used as markers to monitor gene expression patterns of SCs in culture. RNA for SCs was extracted at different timepoints, and it was found that gene expression patterns for both sensory and motor-derived SCs were dysregulated.

Based on the first study, it was hypothesized that due to loss of signaling from the environment, SCs de-differentiated and were unable to maintain their original phenotype. Therefore, if SCs were placed in the presence of the appropriate environmental cues they may differentiate back into their native phenotype. The cues that were evaluated in this thesis were ECM, GFs and the neurotransmitter Ach. In the second study, sensory, motor, and sciatic nerve-derived SCs were transplanted into sciatic nerve-derived ANGs, which have intact ECM, and then used to treat a 14 mm sciatic nerve defect. After 2 weeks, the expression levels of GFs (NGF, GDNF and BDNF) and sensory and motor phenotypic markers were evaluated compared to an acellular graft. The groups with sensory or motor-derived SCs had higher levels of GFs, which are necessary to attract and guide regenerating axons across the defect. However, ECM in a mixed nerve ANG did not promote native phenotypic marker expression of SCs, which suggests that additional cues may need to be included into the graft for further differentiation of SCs into the sensory or motor phenotype.

In the third study, the effects of GFs and Ach on SC differentiation were evaluated. Sensory or motor-derived SCs were cultured with different doses of GFs (sensory and motor) and Ach (motor only). Using a proliferation assay, the number of cells present after a week culture with GFs was determined. The SCs treated with both NGF and GDNF had lower cell numbers after 7 days compared to SCs grown without supplementation, which implies that SCs may have been differentiating toward a more mature phenotype and reached a post-mitotic state. qRT-PCR was used to determine the levels of S100 (differentiated marker) and nestin (undifferentiated marker) after treating SCs with NGF and GDNF. Both GFs promoted increased expression of S100 and decreased nestin in a dose-dependent manner. Further studies also revealed that NGF also further differentiated the

93

sensory-derived SCs by promoting an increase in sensory marker (MBP & BDNF) expression. GDNF promoted the increased expression of sensory markers in sensory-derived SCs and motor markers (PRKCi & VEGF) in motor-derived SCs. This part of the study suggested that NGF and GDNF may be necessary in the SC environment to promote SC differentiation and phenotypic marker expression to myelinate and support the regenerating axons.

The next part of the study studied the effects of Ach on motor-derived SCs. A proliferation assay was used to determine the number of cells present after 7 days of treatment with Ach. The number of cells present after 7 days in the group treated with 0.1 mg/mL of Ach was lower than the groups treated with no Ach or with 0.01 mg/mL Ach. Using qRT-PCR analysis, it was shown that the Ach promoted increased S100 expression as well as increased motor-specific marker expression. The effect of Ach on SCs was further validated by blocking Ach receptors on the SC surface with gallamine, which inhibited the expression of motor markers in SCs treated with Ach. These results suggest that the Ach may be necessary in the SC environment to promote the differentiation of the motor-derived SCs into mature motor marker expressing SCs. Overall this study focused on evaluating different aspects of the SC environment *in vivo* that may contribute to the SC differentiation and phenotypic maintenance. The results from this thesis stress the importance of considering other molecules and factors that are important in designing transplantation therapies.

5.2   Recommendation for Future Directions

This thesis work focused on evaluating environmental cues (ECM, GFs and Ach) that affect the differentiation of SCs. However, the factors contributing to SC

94

differentiation are not limited to the molecules evaluated in this thesis. In the second study, it was shown that the inclusion of phenotype-specific SCs into ANGs promoted GF expression, but did not promote differentiation of the cells into their native phenotypes. This result implies that other factors and cues need to be included along with the SCs in ANGs. Therefore further studies need to be done to build a microenvironment for SCs to design efficient therapies to treat peripheral nerve injury. Furthermore, to prevent the sacrifice of a donor nerve for SC transplantation therapies, alternative sources of SCs need to be explored. In addition, the source of the donor ANGs may also affect the differentiation and expression patterns of phenotypic markers of the SCs as the axons regenerate.

5.2.1 Building a Microenvironment for SC Transplantation Strategies

To transplant a sufficient number of cells at the injury site, the SCs must first be expanded *in vitro*. As SCs are expanded *in vitro*, they undergo various treatments to rid the culture of fibroblasts, which prevent the growth of SCs by overtaking the culture dish. Throughout expansion, the culture is constantly purified to obtain a homogeneous culture of SCs. However, the loss of signaling from axons and the environment de-differentiates SCs into an immature proliferative state. Thus it is important to understand what other cues from the environment besides proteins and small molecule studied in this thesis affect the behavior and differentiation of SCs. Other cues that must be explored include other cells present in the regenerating environment, such as fibroblasts, present with SCs in uninjured nerve as well as expansion cultures in two-dimensions (2-D) versus three-dimensions (3-D).

Recent studies have shown that fibroblasts play a key role in promoting axonal regrowth after peripheral nerve injury. The ephrin-B/EphB2 signaling between fibroblasts

and SCs may direct the migration of SCs from the distal nerve stump to form bands of Bungner to guide axonal regeneration. In addition to promoting the clustering of SCs at the injury site, fibroblasts may have an effect on the differentiation of SCs as axons regenerate (Parrinello, Napoli et al. 2010). Further studies should be done to understand the signaling between the two cells types (SCs and fibroblasts) to determine if fibroblasts have positive effects on SC differentiation into a mature phenotype. If SCs differentiate in response to signaling from fibroblasts, fibroblasts may be a positive addition to cell transplantation therapies for nerve regeneration.

Alternatively, SCs cultured in a 3-D scaffold may have an effect on the differentiation of SCs. The SCs used in this thesis were cultured on poly-L-Lysine (pLL) coated petri dishes, which is a 2-D surface. The expansion of SCs on pLL dishes does not mimic actual cell-cell signaling between SCs *in vivo,* which is a 3-D environment. After expansion in culture, differentiation of SCs in a 3-D environment could be studied by culturing cells in scaffolds, such as collagen (Chamberlain, Yannas et al. 1998; Labrador, Buti et al. 1998) or fibrin (Lee, Yu et al. 2003; Galla, Vedecnik et al. 2004; Marcol, Kotulska et al. 2005). Additionally, these scaffolds can be modified to attach cues (GFs and small molecules) (Sakiyama-Elbert and Hubbell 2000; Sakiyama-Elbert and Hubbell 2000) to further mimic the *in vivo* environment. Therefore, a modified scaffold with GFs, small molecules, and SCs could serve as a platform to deliver necessary cues and cells to promote better nerve regeneration.

5.2.2 Alternative Sources of SCs for Transplantation

The transplantation of SCs at the injury site has been shown to promote nerve regeneration. However, the source of SCs for transplantation needs to be explored further.

Current methods require the sacrifice of a healthy nerve for either autologous SC transplantation or immune-suppression for allogenic (cadaveric typically) SC transplantation. Although transplantation of autologous SCs promoted healthy nerve regeneration, the sacrifice of a healthy nerve from the patient leaves the donor site in a morbid condition. Therefore less invasive methods to obtain cells for transplantation need to be identified.

Recent work in the area has shown the transplantation of postnatal skin-derived precursor SCs (SKP SCs) promotes peripheral nerve regeneration in a rat tibial nerve model (Walsh, Gordon et al. 2010). SKPs are cells derived from the dorsal torso skin and exhibit properties similar to neural crest stem cells (NCSCs). NCSCs arise from the ectoderm during development and differentiate into multiple lineages including peripheral neurons and glia. SKPs are thought to arise during embryogenesis and persist in the dermis layer of the skin through adulthood. SKPs can be harvested from the patient without invasive surgery, and be expanded in culture as proliferating self-renewing spheres (Toma 2001). The cells can be further differentiated into cells expressing SC phenotypic markers (S100, GFAP, and p75) (Toma 2001; Biernaskie, McKenzie et al. 2006) and be used in for transplantation therapies to promote nerve regeneration (McKenzie, Biernaskie et al. 2006). Although it has been shown that SKP SCs can promote regeneration, these transplanted SKP SCs were derived from postnatal rats and transplanted into adult rats (Walsh, Biernaskie et al. 2009). To be clinically relevant, further studies need to be conducted to demonstrate the therapeutic capability of SKP SCs derived from adult sources. In addition, studies showing the types of axons that regenerate, as well as a more detailed assessment of the differentiation of SKP SCs will help in designing less invasive cell transplantation therapies.

5.2.3   Alternative Sources of ANGs

Currently, ANGs derived from any available source are used to treat nerve defects in peripheral nerve injuries. However, the nerve source may play a role in the differentiation and expression patterns of the SCs. In the second study, ANGs derived from the rat sciatic nerve were used as the donor nerves to repair a 14 mm sciatic nerve injury. These ANGs were then transplanted with SCs derived from both motor and sensory nerves, as well as the sciatic nerve. The ANGs supplemented with sciatic-derived SCs tended to have similar expression patterns to the isograft, which is also sciatic-nerve derived. This result suggests that the intact ECM present within the ANG was sufficient for similar expression patterns to the SCs present within the isograft. But the sciatic-nerve derived ANGs failed to provide the necessary cues to differentiate the motor or sensory-derived SCs within the ANGs into their native phenotype. These results combined imply that the intact ECM present within nerve sources may affect the differentiation and phenotypic marker expression of the transplanted SCs.   Therefore, it would be beneficial to study the effects of ANG nerve source on the phenotypic marker expression on immature cultured motor or sensory-derived SCs.

5.3   Concluding Remarks

The overall intention of this thesis was to study different environmental cues that affect the differentiation and phenotype of SCs. Because SCs are necessary for successful peripheral nerve regeneration, understanding which cues affect SC gene expression patterns and behaviors will aide in designing better and more efficient transplantation therapies. This may not only promote axon regeneration, but also promote motor or sensory specific axonal regrowth as well.

Appendix

Table A.2.1: Genes that are upregulated in the motor branch of the femoral nerve versus sensory branch. *M –* average signal intensity in motor nerve group, *S –* average signal intensity in sensory nerve group (*n = 3*)

Gene Name	Gene common name	Accession Number	M/S	std dev/average
amphiphysin 1	Amph1	NM_022217	2.05	0.277
ankyrin repeat domain 6	Ankrd6	BF391635	3.62	0.108
asporin	Aspn	AI639412	2.38	0.023
calsequestrin 2	Casq2	NM_017131	2.19	0.101
cellular retinoic acid binding protein 2	Crabp2	U23407	4.19	0.174
cystathionase (cystathionine gamma-lyase)	Cth	NM_017074	2.22	0.294
endothelial cell-specific molecule 1	Esm1	NM_022604	2.81	0.013
family with sequence similarity 82, member A	Fam82a	AW528443	2.16	0.050
four and a half LIM domains 1	Fhl1	BI298356	2.52	0.227
four and a half LIM domains 2	Fhl2	NM_031677	2.10	0.124
forkhead-like 18 (Drosophila)	Fkhl18	AI008883	2.31	0.052
fucosidase, alpha-L- 2, plasma	Fuca2	BM389993	2.04	0.108
glutathione peroxidase 2	Gpx2	AA800587	3.84	0.015
hypothetical protein LOC307347	LOC307347	AA817959	2.37	0.089
hypothetical protein LOC691750	LOC691750	AI711537	2.08	0.224
Meis1, myeloid ecotropic viral integration site 1 homolog 2 (predicted)	Meis2_predicted	BF405277	2.27	0.308
neurofilament, light polypeptide	Nefl	NM_031783	3.46	0.018

NADPH oxidase 4	Nox4	NM_053524	2.23	0.101
odd Oz/ten-m homolog 2 (Drosophila)	Odz2	BF418058	2.06	0.189
pre-B-cell leukemia transcription factor 1 (predicted)	Pbx1_predicted	BF419639	2.31	0.042
pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	Pcbd1	BF281220	2.36	0.099
procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	Plod2	BI279641	2.09	0.082
peripheral myelin protein 2	Pmp2	AW533483	5.62	0.185
prostaglandin D2 synthase	Ptgds	J04488	2.33	0.099
parathyroid hormone-like peptide	Pthlh	NM_012636	2.08	0.111
similar to 3632451O06Rik protein (predicted)	RGD1310110_predicted	AI501165	2.11	0.096
similar to Gpc6 protein (predicted)	RGD1563063_predicted	BF409344	2.18	0.189
similar to hedgehog-interacting protein (predicted)	RGD1564108_predicted	AI709766	2.08	0.258
similar to Synaptopodin-2 (Myopodin) (predicted)	RGD1564779_predicted	AI547837	2.17	0.094
similar to RIKEN cDNA D330045A20 (predicted)	RGD1566282_predicted	BE112948	2.28	0.196
similar to RIKEN cDNA D330045A20 (predicted)	RGD1566282_predicted	AW142796	2.82	0.116
ring finger protein 139 (predicted)	Rnf139_predicted	BE121079	2.01	0.046
sulfotransferase family 1D, member 1	Sult1d1	NM_021769	2.15	0.047
tissue factor pathway inhibitor 2	Tfpi2	AI179507	2.63	0.241
transmembrane protein 132E	Tmem132e	AW527684	2.19	0.057
troponin T2, cardiac	Tnnt2	NM_012676	2.88	0.072
tubulin, beta 2b	Tubb2b	X03369	2.03	0.170

ubiquitin carboxy-terminal hydrolase L1	Uchl1	NM_017237	2.26	0.016

Table A.2.2: Genes that are upregulated in the sensory branch of the femoral nerve versus motor branch. $M-$ average signal intensity in motor nerve group, $S-$ average signal intensity in sensory nerve group ($n=3$)

Gene Name	Gene common name	Accession Number	S/M	std dev/average
adiponectin, C1Q and collagen domain containing	Adipoq	BM386227	26.19	0.262
adrenomedullin receptor	Admr	BF551274	12.08	0.121
aldolase C	Aldoc	NM_012497	25.63	0.256
ankyrin 3	Ank3	AJ428573	1.70	0.017
ankyrin 3	Ank3	BF398752	24.24	0.242
ankyrin 3	Ank3	BF392810	7.90	0.079
ankyrin 3	Ank3	BF393943	16.83	0.168
axin2	Axin2	NM_024355	12.36	0.124
complement component 4 binding protein, alpha	C4bpa	NM_012516	20.64	0.206
carbonic anhydrase 3	Ca3	AB030829	2.01	0.020
carbonic anhydrase 3	Ca3	NM_019292	22.43	0.224
carbonic anhydrase 3	Ca3	AW144120	22.19	0.222
coiled-coil domain containing 37 (predicted)	Ccdc37_predicted	BE121191	27.23	0.272
cadherin 2	Cdh2	NM_031333	4.83	0.048
cysteine dioxygenase 1, cytosolic	Cdo1	NM_052809	27.61	0.276
complement factor D (adipsin)	Cfd	AI237358	32.18	0.322
chromodomain helicase DNA binding protein 7 (predicted)	Chd7_predicted	AI599104	18.06	0.181
chondrolectin (predicted)	Chodl_predicted	AI029745	16.51	0.165

procollagen, type XI, alpha 1	Col11a1	AI136248	0.38	0.004
chemokine (C-X-C motif) ligand 14	Cxcl14	BG380414	20.92	0.209
deoxyguanosine kinase (predicted)	Dguok_predicted	AI599463	1.77	0.018
endothelin converting enzyme 1	Ece1	BE107816	7.47	0.075
endonuclease G	Endog	AW253957	3.57	0.036
coagulation factor II (thrombin) receptor-like 2	F2rl2	BG671473	12.77	0.128
coagulation factor V	F5	AI717113	4.57	0.046
fidgetin (predicted)	Fign_predicted	BE098265	5.83	0.058
FXYD domain-containing ion transport regulator 7	Fxyd7	NM_022008	5.05	0.051
G0/G1 switch gene 2	G0s2	AI406939	0.33	0.003
glycerol-3-phosphate dehydrogenase 1 (soluble)	Gpd1	BF399697	12.56	0.126
glycerol-3-phosphate dehydrogenase 1 (soluble)	Gpd1	BI277042	31.56	0.316
glycoprotein m6b	Gpm6b	BE096035	22.80	0.228
G protein-coupled receptor 37-like 1	Gpr37l1	AF087947	10.80	0.108
G protein-coupled receptor 56	Gpr56	AI412938	13.48	0.135
glutamate receptor, ionotropic, kainate 2	Grik2	NM_019309	7.86	0.079
immunoglobulin superfamily, member 11	Igsf11	BI298836	11.29	0.113
immunoglobulin superfamily, member 4A	Igsf4a	BE117767	32.35	0.323
potassium voltage gated channel, Shal-related family, member 3	Kcnd3	NM_031739	33.99	0.340

L1 cell adhesion molecule	L1cam	NM_017345	8.22	0.082
leucine-rich repeat LGI family, member 4	Lgi4	AI058424	16.90	0.169
lipase, hormone sensitive	Lipe	NM_012859	5.24	0.052
common salivary protein 1	LOC171161	NM_133622	11.09	0.111
	LOC360570	BF281984	21.97	0.220
adenosine monophosphate deaminase 2 (isoform L)	LOC362015	BE100752	22.02	0.220
zinc finger protein 467	LOC500110	BF417765	6.54	0.065
cell death-inducing DFFA-like effector c	LOC500292	AA818135	11.31	0.113
leucine rich repeat containing 4B (predicted)	Lrrc4b_predicted	BF546934	12.23	0.122
microtubule-associated protein tau	Mapt	BE107978	33.45	0.334
myelin basic protein	Mbp	BE109730	15.88	0.159
neural cell adhesion molecule 1	Ncam1	AW529710	28.28	0.283
neural cell adhesion molecule 1	Ncam1	AI409738	17.29	0.173
neuroligin 1	Nlgn1	BF400127	33.74	0.337
neuroligin 3	Nlgn3	NM_134336	30.03	0.300
nuclear receptor subfamily 4, group A, member 2	Nr4a2	U72345	22.56	0.226
neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	BE102996	29.16	0.292
neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	BG669126	25.83	0.258
nucleotide binding protein 2	Nubp2	BF397271	3.72	0.037
purinergic receptor P2X, ligand-gated ion channel, 7	P2rx7	AI385229	10.28	0.103

protocadherin 17 (predicted)	Pcdh17_predicted	BF558981	11.60	0.116
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374	28.58	0.286
platelet derived growth factor receptor, beta polypeptide	Pdgfrb	AI071374	3.51	0.035
perilipin	Plin	NM_013094	27.99	0.280
plasmalemma vesicle associated protein	Plvap	NM_020086	5.31	0.053
protein kinase, cAMP dependent regulatory, type II beta	Prkar2b	M12492	15.04	0.150
protein tyrosine phosphatase, non-receptor type 3	Ptpn3	BF403190	24.41	0.244
sterol regulatory element binding factor 1	Rai1_predicted	AF286470	13.15	0.131
RAS, dexamethasone-induced 1	Rasd1	AF239157	3.45	0.035
Ras association (RalGDS/AF-6) domain family 4	Rassf4	AI227769	25.84	0.258
similar to hypothetical protein FLJ31846 (predicted)	RGD1306118_predicted	AI231461	32.63	0.326
similar to Protein C22orf5	RGD1306591	AI577870	8.80	0.088
similar to AT motif-binding factor (predicted)	RGD1560268_predicted	BF396082	0.96	0.010
similar to homeotic protein Hox 2.2 - mouse (predicted)	RGD1562142_predicted	BF396436	11.80	0.118
similar to RIKEN cDNA 6330512M04 gene (predicted)	RGD1563319_predicted	BI303199	8.75	0.088
similar to C-type lectin-like receptor 2 (predicted)	RGD1563517_predicted	AI029991	11.25	0.113
similar to Serologically defined colon cancer antigen 13 (predicted)	RGD1564816_ predicted	BF416289	15.95	0.159

ryanodine receptor 3	Ryr3	AI072862	14.81	0.148
S100 calcium binding protein A9 (calgranulin B)	S100a9	NM_053587	13.67	0.137
secretory carrier membrane protein 1	Scamp1	AI013502	9.66	0.097
secretory carrier membrane protein 1	Scamp1	AW524864	14.76	0.148
scavenger receptor class B, member 1	Scarb1	NM_031541	7.08	0.071
septin 5	Sep5	NM_053931	32.47	0.325
sterol regulatory element binding factor 1	Srebf1	BF398848	4.76	0.048
sperm specific antigen 2 (predicted)	Ssfa2_predicted	BF400722	32.57	0.326
stefin A2 (predicted)	Stfa2_predicted	BF415134	25.09	0.251
thyroid hormone responsive protein	Thrsp	NM_012703	3.70	0.037
thyroid hormone responsive protein	Thrsp	AI169092	6.19	0.062
thyroid hormone responsive protein	Thrsp	NM_012703	16.67	0.167
tweety homolog 1 (Drosophila) (predicted)	Ttyh1_predicted	BM387179	23.67	0.237
tumor suppressor candidate 5	Tusc5	BM386662	30.28	0.303

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Vita

Nithya Jothi Jesuraj

Date of Birth	September 20, 1985
Place of Birth	Framingham, MA
Degrees	**Ph.D. Biomedical Engineering**
	Washington University, St. Louis, Missouri, May 2012
	M.S. Biomedical Engineering
	Washington University, St. Louis, Missouri, Dec 2010
	B.S. Chemical Engineering
	Cornell University, Ithaca, New York, May 2007
Professional Societies	Biomedical Engineering Society
	Society for Biomaterials

Journal Publications

Jesuraj NJ, Santosa KB, Newton P, Liu Z, Hunter DA, Mackinnon SE, Sakiyama-Elbert SE, Johnson PJ, A Systematic Evaluation of Schwann Cell Injection into Acellular Cold-Preserved Nerve Grafts, J. Neurosci Methods. 2011 April: 197 (2): 209-215.

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Santosa KB, **Jesuraj NJ,** Valls AV, MacEwan MR, Newton P, Hunter DA, Mackinnon SE, Johnson PJ, Effects of Supplementation of Acellular Nerve Allografts with Schwann Cells Transfected with Glial-Cell Line Derived Neurotrophic Factor on Peripheral Nerve Regeneration (Submitted)

Jesuraj NJ, Kwasa JA, Sakiyama-Elbert SE, Glial-derived neurotrophic factor promotes increased phenotypic marker expression in femoral sensory and motor-derived Schwann cell cultures (Submitted)

Namani R, Feng Y, Okamoto RJ, **Jesuraj NJ**, Sakiyama-Elbert SE Genin GM, Bayly PV, Elastic Characterization of Transversely Isotropic Soft Materials by Dynamic Shear and Asymmetric (In Press)