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

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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 v2.0 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.

Keywords

nerve regeneration; cell transplantation; peripheral nerve injury

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

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(Burnett and Zager 2004; Schmidt and Leach 2003). 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 et al. 1993; Hare et al. 1995; Levi et al. 1994). 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 et al. 1982; Sondell et al. 1998; Whitlock et al. 2009). However, the regenerative capacity of ANGs is still inferior to autografts because they lack SCs (Whitlock et al. 2009). SCs provide GFs and ECM to promote neuronal survival and axonal regeneration (Brenner et al. 2005; Bunge 1993; Frostick et al. 1998; Nagarajan et al. 2002; Reynolds and Woolf 1993). 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 et al. 1996; Madison et al. 1999; Madison et al. 2007; Madison et al. 2009). Previous studies have shown that sensory axons may also regenerate preferentially down sensory pathways (Hoke 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 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 1993; Bunge 1994; Bunge et al. 1986). 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 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*.

MATERIALS AND METHODS

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 RNAlater® 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 $\mu\text{g}/\mu\text{L}$) 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 et al. 1997). The extracted RNA was used for further experiments. Since the majority of the nerves are composed of SCs (~90%) (Oda et al. 1989), the harvested RNA was assumed to be representative of the SC RNA present in fresh nerve tissue.

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 μ g) 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 μ g) 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 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.

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. 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 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).

SC Culture Preparation for Time Study

SC cultures were prepared as previously described (Pruss 1982; Raff et al. 1978). 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 $130 \times g$ 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 $\mu\text{g}/\text{mL}$ 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 each gene in freshly harvested femoral motor and sensory nerves.

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 \pm standard deviation.

RESULTS

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 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 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 I). 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 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 II). Neurologin 1 (NLGN1) is a component of the myelin sheath made by SCs (Jahn et al. 2009; Song et al. 1999). 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 et al. 1971) of axons, increased intracellular progesterone in SCs, (Guennoun et al. 2001; Robert et al. 2001), increases the expression of MBP in sensory SCs, thus making MBP a good marker for sensory SCs.

Verification of genes using qRT-PCR

For further analysis, a set of genes was selected from the gene chip analysis that have been shown in previous studies to be involved in either sensory or motor function development and growth: neurologin 1 (NLGN1, sensory), myelin basic protein (MBP, sensory), protein kinase C iota (PRKCi, motor), and neurofilament (NEFL, motor) (Biswas et al. 2010; Roberson et al. 1992; Robert et al. 2001; Tisdale 2000). 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 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 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), 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.

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 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 III), and the expression levels of the genes (Table IV) 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 et al. 2006).

Those genes that showed significant changes in gene expression over 30 days were MBP, NEFL, PTN and PRKCi (Figure 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 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 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 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 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*.

DISCUSSION

A number of studies have shown that the transplantation of SCs at the injury site improves the regeneration of peripheral nerves (Brenner et al. 2005; Fox 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 nonmyelinated 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 (Castro et al. 2008; Schmalbruch 1986). 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 (Jessen and Mirsky 2005; Mirsky and Jessen 1996). 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 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 et al. 2006). These five genes may not have been identified from the gene chip analysis due to the stringent criteria (Costigan 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 1 & 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 (Biswas et al. 2010; Guennoun et al. 2001; Jahn et al. 2009; Robert et al. 2001; Song et al. 1999). 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 (Lariviere and Julien 2004; Mamidipudi and Wooten 2002; Roberson et al. 1992; Tisdale 2000). The remaining two genes, VEGF and PTN, were chosen from literature because they are predominantly expressed in the ventral root after denervation (Hoke 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 IV) 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 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 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 et al. 2001; Robert 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 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 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 (Mamidipudi and Wooten 2002; Tisdale 2000; van Niel 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 et al. 2002; Jin 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 III) to eliminate fibroblasts and to promote the proliferation of the cells using mitogenic supplements. Previously, it has been shown that de-differentiation of SCs may be linked to proliferation (Guertin et al. 2005). However, recent studies have revealed that SC de-differentiation is independent of mitogenic signaling and also uncoupled to proliferation (Monje 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), the 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 et al. 2001; Iwase et al. 2005), neurotransmitters (Vrbova et al. 2009), or supporting cells such as fibroblasts (Parrinello 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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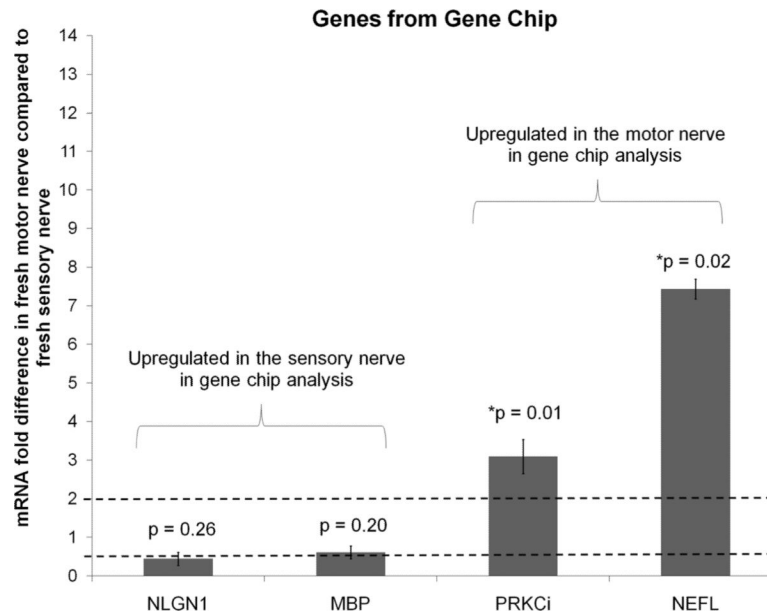


Figure 1. Verification of gene chip trend with qRT-PCR

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 $\Delta C_t(\text{motor} - \beta\text{-actin})$ and $\Delta C_t(\text{sensory} - \beta\text{-actin})$

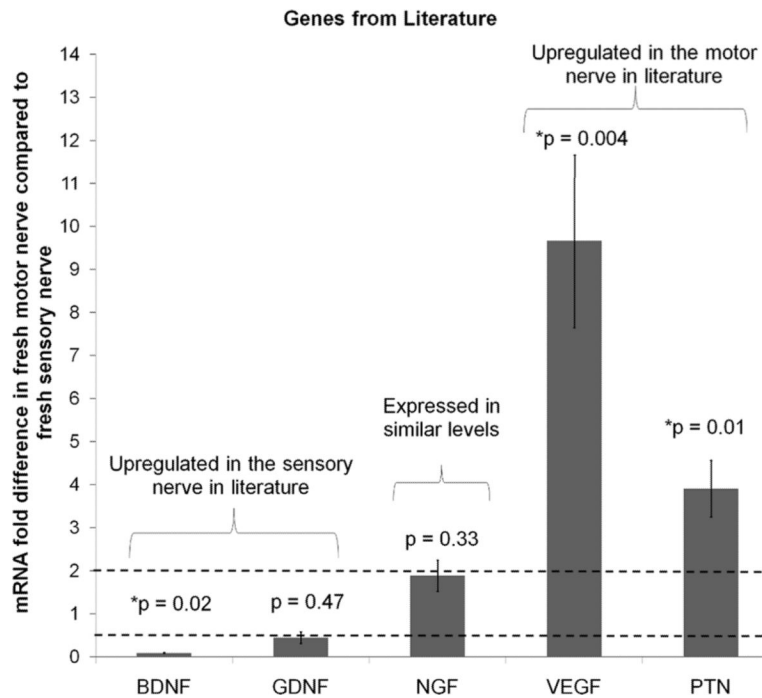


Figure 2. Expression of genes previously identified as markers for sensory and motor SCs
 The expression level of genes that were reported in the literature to be differentially regulated in the motor and sensory SCs (Hoke et al. 2006) was determined by qRT-PCR. The values were normalized to β -actin, 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 $\Delta C_t(motor - \beta-actin)$ and $\Delta C_t(sensory - \beta-actin)$

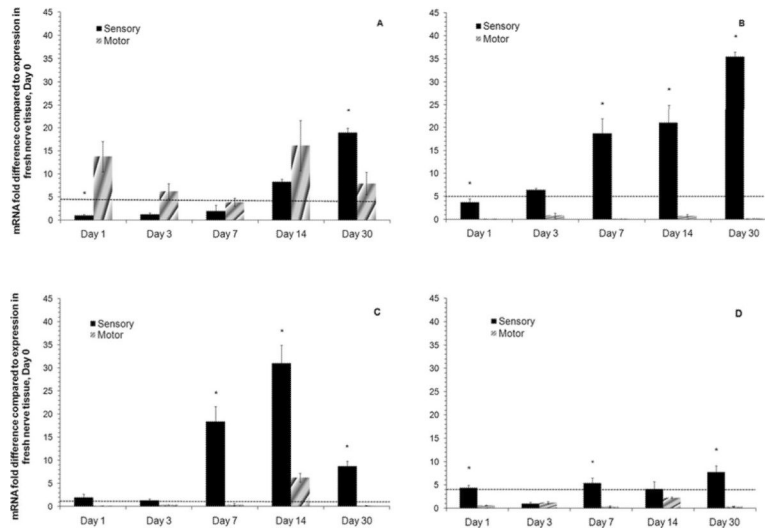


Figure 3. Gene expression in motor and sensory SCs over 30 days of *in vitro* culture by qRT-PCR A) MBP B) NEFL C) PTN and D) PRKCi. The gene expression for each time point was normalized to β -actin, and then the fold difference versus fresh tissue (Day 0) was calculated. The error bars represent the 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.

Table I

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 II

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
Neurologin 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	Pdgfrb	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

Table III

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 IV

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	Neuroigin 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)