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Publication Date	2017-03-10
Publication Information	Chen, Bingkun K., Madigan, Nicolas N., Hakim, Jeffrey S., Dadsetan, Mahrokh, McMahon, Siobhan S., Yaszemski, Michael J., & Windebank, Anthony J. (2018). GDNF Schwann cells in hydrogel scaffolds promote regional axon regeneration, remyelination and functional improvement after spinal cord transection in rats. Journal of Tissue Engineering and Regenerative Medicine, 12(1), e398-e407. doi: 10.1002/term.2431
Publisher	Wiley
Link to publisher's version	https://doi.org/10.1002/term.2431
Item record	http://hdl.handle.net/10379/15321
DOI	http://dx.doi.org/10.1002/term.2431

GDNF Schwann cells in hydrogel scaffolds promote regional axon regeneration, remyelination and functional improvement after spinal cord transection in rats

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Keywords: spinal cord injury, axonal regeneration, GDNF, Schwann cell, axon myelination, hydrogel scaffolds

Abstract

Positively-charged oligo[poly(ethylene glycol)fumarate] (OPF$^+$) is a biodegradable hydrogel used in combinational approaches for spinal cord injury repair. Multichannel OPF$^+$ scaffolds loaded with primary Schwann cells (SCs), or Schwann cells genetically modified to secrete glial cell-derived neurotrophic factor (GDNF-SCs), were implanted into completely transected rat spinal cords. GDNF-SCs promoted the regeneration of higher numbers of axons into OPF$^+$ scaffolds than primary SCs after 4 weeks. This was most significant in central and ventral midline channels of the scaffold. The number of spinal interneurons that projected rostrally through the scaffold was ten-fold higher in the GDNF-SC group. Increased myelination of regenerating axons was observed in the GDNF-SC group. Myelinating cell and axon complexes were formed by Schwann cells from the host animal, and not by implanted cells or host oligodendrocytes. Transected animals transplanted with GDNF-SC OPF$^+$ scaffolds partially recovered locomotor function at weeks 3 and 4 following surgery.

Highlights

- Implantation of GDNF-SC OPF$^+$ scaffolds in a complete transection model improved the number of regenerating axons over implantation of OPF$^+$ scaffolds with primary SCs.

- GDNF-SCs promoted the growth of ascending intraspinal motor neurons as well as anatomically significant centralized and ventral midline axonal regeneration.

- The implantation of GDNF-SCs enhanced the myelination of regenerating axons by host animal Schwann cells and not by oligodendrocytes.

- Phenotypic patterns of premyelination, axon bundling, and mature myelination in OPF$^+$ scaffolds were observed.

- Completely transected animals implanted with GDNF-SC OPF$^+$ scaffolds recovered partial locomotor function.

1.   Introduction

Several key factors limit endogenous repair and neurologic recovery after spinal cord injury. Areas of immune-mediated tissue destruction, cysts, gliosis and adjacent cord atrophy (Bodley, 2002; Quencer and Bunge, 1996) form physical gaps and barriers to neural regeneration (Beattie et al., 1997). The extracellular matrix of the injury site lacks necessary guidance cues to direct axon extension. There are insufficient concentrations of trophic factors needed to promote cell survival and axon outgrowth (Giger et al., 2010) through a structurally chaotic injury field that contains inhibitory molecular cues, including myelin debris and chondroitin sulfate proteoglycans (Kwok et al., 2014). Multiple, concurrently-applied strategies in such a setting are therefore necessary to facilitate repair of the spinal cord. Strategies for neuroprotection and immunomodulation may maximize the ability of surviving tissue to contribute to repair by local plasticity, and are needed in conjunction longer distance axonal regeneration (Fortun et al., 2009). Getting axons into the right places needs further refinement in order to impact upon neurologic function, including strategies to improve the efficiency of signal conduction through myelination and meaningful synapse formation (Zhao and Fawcett, 2013).

Polymer scaffold technologies may be useful in multimodal approaches for the investigation and treatment of spinal cord injury. Surgically implanted polymers can bridge the gaps, form approximations of viable tissue with structured order and serve as a matrix to guide axonal growth through the area of injury, in addition to being architectural systems for the delivery of cellular and molecular therapies (Gamez Sazo et al., 2012; Madigan et al., 2009). Our recent work has focused on the implantation of cell-loaded, multichannel scaffolds fabricated from the positively-charged polymer oligo[poly(ethylene glycol)fumarate] (OPF+) (Chen et al., 2011b; Madigan et al., 2014). Photo-crosslinked OPF+

forms a soft, porous, biodegradable hydrogel with biomechanical properties similar to spinal cord tissue (Dadsetan et al., 2007). We have developed an animal model of severe spinal cord injury using complete transection plus scaffold placement that provides a platform to control the microenvironment of regenerating axons and to make quantitative comparisons between experimental groups. Multichannel OPF[+] scaffolds loaded with primary Schwann cells improved axonal regeneration density and accuracy of growth orientation in comparison to cell-loaded scaffolds fabricated from uncharged OPF, poly(lactic co-glycolic acid) (PLGA), and poly(3-caprolactone fumarate)(PCLF) (Chen et al., 2011a). We have also used the multichannel scaffold model to compare the influence of various implanted cell types, including Schwann cells, mesenchymal stromal cells (MSCs) (Madigan et al., 2014; Rooney et al., 2011) and neural stem cells (Olson et al., 2009). Schwann cells were demonstrated to be consistently superior in supporting axonal regeneration in this model. This role for Schwann cells in spinal cord regeneration supports the observations of others (Takami et al., 2002; Xu et al., 1997) who have introduced Schwann cells for repair in human SCI (Bunge and Wood, 2012).

We have previously demonstrated feasibility of polymeric delivery of neurotrophic factors to the injured spinal cord. We have used mesenchymal stromal cells that were genetically modified by adenovirus to secrete nerve growth factor (NGF) from a poly(lactic-co-glycolic acid) (PLGA) substrate (Rooney et al., 2008). Neurotrophin-3 (NT-3) released from non-viral polyplexes incorporated into an acellular, multichannel collagen scaffold with nanofibers improved *in vivo* axonal regeneration after complete spinal cord transection (Yao et al., 2013). Glial cell-derived neurotrophic factor (GDNF) has been delivered to the injured cord both from polymeric substrates (Iannotti et al., 2003) and from gene-modified fibroblast cell lines seeded within polymer scaffolds (Blesch and Tuszynski, 2003). GDNF is of particular interest for central nervous system repair, having

originally been identified as a survival factor for multiple neuronal populations including midbrain dopaminergic neurons, locus ceruleus noradrenergic neurons (Sariola and Saarma, 2003), spinal ganglionic sensory neurons (Bennett et al., 1998) and corticospinal motor neurons (Junger and Varon, 1997). Polymeric delivery of Schwann cells overexpressing GDNF has been shown to enhance neuronal survival, axonal regeneration, remyelination and synaptic remodeling after hemisection spinal cord injury (Deng et al., 2013).

Our objective in this study was to compare the regenerative capacity of OPF[+] scaffolds containing primary unmodified Schwann cells (SCs) to OPF[+] scaffolds delivering Schwann cells that have been genetically modified by retrovirus to secrete a high concentration of GDNF (GDNF-SCs). We compare the number of regenerating axons within each scaffold type at a four week time point after complete spinal cord transection injury. We determine whether the implantation of GDNF-SC OPF[+] scaffolds influences the topographic distribution of regeneration through scaffold channels that are adjacent to specific spinal cord tracts. Retrograde axon tracing is used to demonstrate the rostral-caudal directionality and density of neuronal growth through the scaffold into the distal cord. Our initial hypothesis had been that GDNF, a neurotrophic factor associated primarily with motor neuron survival, might preferentially influence the regeneration of descending motor tracts. The extent to which regenerating axons are myelinated in SC OPF[+] and GDNF-SC OPF[+] implanted animals is analyzed by further defining a developmental relationship between axons and host animal Schwann cells. Finally, we assess the ability of completely transected animals transplanted with GDNF-SC OPF[+] scaffolds to recover hindlimb locomotor function, which had not yet been observed in prior studies in our model.

2. Materials and Methods

2.1. Retroviral Expression Vectors

pLXSN-IRES-eGFP was provided by Dr. Thomas Ritter at the Regenerative Medicine Institute, National University of Ireland, Galway. This construct was cloned as a hybrid of two commercially available plasmids. The retroviral expression vector pLXSN (Invitrogen, Carlsbad CA USA) contains 5' and 3' Moloney murine sarcoma virus long terminal repeats (LTR), the Ψ^+ extended viral packaging signal, and a neomycin antibiotic selectivity gene driven by an early SV40 promotor. pIRES2-eGFP (Invitrogen) contains an internal ribosomal entry site (IRES2) derived from the encephalomyocarditis virus (ECMV) and the enhanced green fluorescent protein (eGFP) coding region (Cormack et al., 1996). A 2040 basepair EcoRI-Hpa1 cassette was excised from pIRES-eGFP and ligated into pLSXN to construct pLXSN-IRES-eGFP. Human GDNF (variant 1) cDNA was also kindly provided by Dr. Thomas Ritter. The GDNF sequence was amplified using PCR primers to create appropriate restriction sites and to incorporate the Kozak ribosomal targeting sequence (Kozak, 1990). Primers (5' forward ACGCGTGCCACCATGAAGTTATGGGATGTC, 3' reverse GTCGACTCAGATACATCCACACCT) were custom synthesized by Eurofins MWG/Operon, Ebersberg, Germany. GDNF amplicons were subcloned into a shuttle vector (pCRII-TOPO, Invitrogen) for banking and sequencing prior to ligation to construct pLXSN-GDNF-IRES-eGFP. GDNF gene inserts were re-sequenced, and verified by BLAST alignments to the source sequences.

Ecotropic, replication-incompetent retrovirus for GDNF and eGFP gene delivery to Schwann cells was produced by transfecting 25 μg of pLXSN-GDNF-IRES-eGFP DNA into GP+E86 NIH-3T3 packaging cells (Markowitz et al., 1990) using jetPEI™ (PolyPlus

Transfection, Illkirch, France), a linear polyethylenimine (PEI) derivative for cationic gene delivery. GP+E86 cells were sub-confluently seeded at 80,000 cells per ml on 10 cm dishes in growth medium (Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco, Carlsbad CA USA)). On Day 4 new GP+E86 cells were plated at the same density and were pre-treated for 16 hours with 100 ng/ml tunicamycin (Sigma-Aldrich, St. Louis, MO USA). On Day 5, supernatant media containing a low titer retrovirus was removed from the transfected packaging line, filtered and transferred to new tunicamycin pre-treated packaging cells in the presence of 8 μg/ml of polybrene (1.5-dimethyl-1.5-dizaundecamethylene polymethobromide (Sigma-Aldrich)) for 24 hours. Virally-transduced packaging cells were selected in growth media containing 1 mg/ml G418 neomycin analogue (Sigma-Aldrich) for 10 days and then maintained in growth media alone to produce high titer retroviral supernatant media.

2.2. Primary and GDNF-Secreting Schwann Cells

Primary rat Schwann cells were cultured from the sciatic nerves of two to five day old newborn Sprague-Dawley pups as we have described (Chen et al., 2009; Chen et al., 2011b; Olson et al., 2009). To produce GDNF-secreting, eGFP-positive Schwann cells (GDNF-SC), early-passage primary Schwann cells were seeded at a density of 80,000 cells per ml and grown under filtered high titer (2×10^{10} cfu) retroviral supernatant media in the presence of 8 ug/ml polybrene for 24 hours. Transduced Schwann cells then were selected for 12 days with 1 mg/ml G418 analogue in Schwann cell media (50:50 DMEM:F12 media containing 10% FBS, 1% antibiotic-antimycotic), following which they were expanded in culture for use. The phenotypic purity of SC and GDNF-SC preparations was determined by immunohistochemical analysis. 20,000 cells in quadruplicate were seeded onto laminin-coated coverslips, and were fixed in phosphate buffered saline (PBS) with 4%

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paraformaldehyde after 24 hours. The cells were incubated with primary antibodies to the S-100 antigen (mouse anti-rat, 1:300, Biogenex San Ramon CA USA), eGFP (rabbit IgG polyclonal, 1:400, MBL International, Woburn, MA, USA) and GDNF (goat anti-rat, 1:400, R&D Systems, Minneapolis, MN, USA). Cells were counted in 10 randomly selected 10x magnification fields to determine the percentage of DAPI+ nucleated cells that co-localized with the antibody labelling. 98.37 ± 0.02 % of cells were S-100 positive in wild type SCs at passage 2. The cell population of GDNF-SCs stained homogeneously (100%) for S-100, eGFP, and GDNF following antibiotic selection (Figure S1).

2.3. *In Vitro* Measurement of GDNF Secretion

The concentration of GDNF secreted *in vitro* from wild type SCs and GDNF-SCs was measured at 24 hour and 72 hour time points from 2.5×10^5 cells, which were either plated as a cell monolayer or loaded into OPF+ scaffolds in quadruplicate. Growth factor concentrations within the supernatant cell culture media were assayed by ELISA (R&D Systems, Minneapolis MN USA) against a four parameter logistic standard curve, according to the manufacturer's protocol. Streptavidin-HRP conjugation was amplified using the ELAST® biotinyl tyramide system (PerkinElmer, Waltham, MA, USA) to measure the low levels of secretion from wild type SCs. Values were calculated as mean +/- SEM, and one-way ANOVA was used determine significant differences in mean GDNF concentration.

2.4. OPF+ Scaffold Fabrication

Positively charged OPF+ scaffolds were fabricated as we have previously described (Chen et al., 2011a; Madigan et al., 2014). OPF macromer was synthesized as a condensation reaction between poly(ethylene glycol) (PEG) and triethyamine (Jo et al., 2000) and dissolved in deionized water containing 0.05% (w/w) of photoinitiator (Irgacure 2959,

Ciba-Specialty Chemicals) and 0.3 g of the cross-linking reagent N-vinyl pyrrolidinone (NVP). OPF was chemically modified at 20% w/w with the positively charged monomer [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) (80% wt in water, Sigma-Aldrich) (Dadsetan et al., 2009). MAETAC is a bifunctional molecule containing a free pH-independent cationic tertiary ammonium group and a reactive methacryloyl group which becomes crosslinked. OPF+ hydrogel scaffolds were fabricated by mold injection of liquid polymer, cast over seven parallel wires of 290 μm diameter and polymerized by exposure to UV light (365 nm) at an intensity of 8 mW/cm² (Black-Ray Model 100 AP). Individual scaffolds were cut into two millimeter lengths and sterilized prior to cell loading by immersion in serial dilutions of ethanol. Scaffolds were vacuum-dried for 24h to remove any residual ethanol, then sealed in sterilized glass vials and stored desiccated at 4°C until further use upon rehydration in cell culture medium.

2.5. OPF+ Scaffold Cell Loading and Surgical Implantation

Wild type SC and GDNF-SC cultures were resuspended in Matrigel™ (BD Biosciences) at a density of 100,000 cells/μL and loaded to 476,000 cells per scaffold (68,000 cells per channel of 0.67 μl volume). Loading efficiency was confirmed by immediately flushing out cells and counting in a hemocytometer for a sample of scaffolds. Surgical materials and full methodology for scaffold implantation was as we have previously described (Chen et al., 2011b; Madigan et al., 2014). Laminectomy through the T8-T10 level, complete T9 spinal cord transection and scaffold implantation were performed on female Sprague-Dawley rats weighing 230-300 grams (Harlan Laboratories, Madison WI USA) in six experimental groupings of 10 animals per group. Three groups of animals received OPF+ scaffolds loaded with wild type primary SCs, and three groups received OPF+ scaffolds loaded with GDNF-

SCs. For each scaffold cell type, one group of 10 animals was designated for neurofilament staining and axon counting, and two groups of 10 animals were designated for Fast Blue retrograde tracing. All animals were cared for with 24 hour availability of technicians and veterinarians experienced in the management of spinal cord injury in rodents. All surgical and care procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic, Rochester MN USA, in accordance with the National Institute of Health, and the Institute for Laboratory Animal Research (ILAR) within the National Academy of Sciences (National Academy of Sciences, 2010).

2.6. Fast Blue Retrograde Axonal Tracing

Two groups in each of the wild type and GDNF-SC animals underwent a second surgery three weeks after scaffold implantation for Fast Blue (FB) retrograde tracing analysis, as we have previously described (Chen et al., 2009). The surgical site was re-exposed and 0.6 µl of 5% aqueous FB (EMS-Chemie GmbH, Groß-Umstadt, Germany) was stereotactically injected into the thoracic spinal cord 5 mm rostral (10 animals) or 5 mm caudal (10 animals) to the implanted scaffold, using a 26 gauge needle attached to a 10-µl Hamilton syringe (Reno, NV, USA). All injections were performed over 30 seconds and the injection needle was kept in place for additional 30 seconds to minimize leakage on withdrawal. The injection site was observed for heavy dye uptake following tissue sectioning to ensure that the dye had been correctly injected. The FB tracing methodology was validated in control animals whose spinal cord was not transected, as we have previously described (Chen et al., 2009).

2.7. Tissue Preparation and Sectioning

Four weeks after scaffold implantation, animals were humanely euthanized by deep anesthesia and transcardial perfusion with either 4% paraformaldehyde in PBS for tissue paraffin-embedding or with 4% paraformaldehyde plus 10% sucrose for tissue cryo-embedding. The spinal column and cord with the scaffold implants were removed *en bloc* and post-fixed overnight in the same fixative at 4°C prior to dissecting free the entire spinal cord length. For neurofilament axon counting groups, the spinal cord segments were embedded in paraffin and cut transversely into 8-μm sections on a Reichert-Jung Biocut microtome (Leica, Bannockburn, IL USA). The ventral-dorsal orientation of the spinal cord and scaffold was carefully preserved during tissue processing and sectioning to allow for identification of three ventral and three dorsal channels surrounding the central scaffold channel. For FB-tracing groups, the tissue was transferred to PBS with 15% sucrose 24 hours at 4°C before being processed for cryostat embedding with Tissue Freezing Medium (TFM) (TBS, Triangle Biomedical Sciences, Durham NC USA). The spinal cord length was cut into 15 mm segments designated P1, P2 and P3 moving rostrally, and S1, S2 and S3 in caudal segments. The P1 segment contained the OPF+ scaffold and FB-injection site. These spinal cord segments were cut longitudinally into 30 μm sections (Reichert HistoSTAT Cryostat Microtome).

2.8. Immunohistochemistry

Primary antibodies were used against axon neurofilament protein (NF) (mouse anti-rat monoclonal antibody, 1:50, Dako, Carpinteria CA USA); Tuj-1 (B-III tubulin) (mouse anti-rat, 1:300, Millipore Chemicon, Temecula CA USA); choline acetyltransferase (CAT) (goat anti-rat, 1:50, Millipore); glial fibrillary acid protein (GFAP) (rabbit anti-rat, 1:100, Dako); myelin basic protein (MBP) (goat anti-rat, 1:400, Santa Cruz Biotechnology, Dallas TX USA);

myelin protein P0 (rabbit anti-rat, 1:2000, Millipore); Olig-2 (rabbit anti-rat, 1:400, Millipore); S-100 antigen (mouse anti-rat, 1:300, Biogenex San Ramon CA USA); p75 neurotrophin receptor (rabbit anti-rat, 1:800, Promega, Madison WI, USA); eGFP (rabbit IgG polyclonal, 1:400, MBL International, Woburn, MA, USA); and GDNF (goat anti-rat, 1:400, R&D Systems, Minneapolis MN USA). Secondary antibodies included biotinylated anti-mouse IgG (1:100, Dako) (Neurofilament); Cy-2 AffiniPure donkey anti rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove PA, USA) (eGFP, p75); Cy3-conjugated affinity purified donkey anti-rabbit IgG (1:200, Millipore Chemicon) (GFAP, Olig-2, P0); Cy3-conjugated affinity purified donkey anti-mouse IgG (1:200, Millipore Chemicon) (B-III tubulin); AlexaFluor™ 647-conjugated AffiniPure donkey-anti goat IgG (1:200, Jackson ImmunoResearch Laboratories) (GDNF, MBP, CAT). Antibody-conjugated horseradish peroxidase was bound to streptavidin (ExtrAvidin-Peroxidase™, Sigma-Aldrich) and 3,3'-diaminobenzidine (DAB) was used as the tertiary chromogen for neurofilament staining.

Rehydrated paraffin-embedded tissue sections were permeabolized in PBS with 0.4% Triton X-100. Slides were immersed in 1 mM EDTA in PBS with 0.05% Tween 20, pH 8.0, heated to 96°C for antigen retrieval and blocked with 10% normal donkey serum in PBS with 0.1% Triton. Sections were incubated with primary antibody, diluted into 5% normal donkey serum in PBS with 0.3% Triton X-100, in a humidified slide chamber overnight at 4°C followed by serial washing (PBS with 0.1% Triton X-100) and application of secondary antibody. For antibody staining that co-localized with FB dye, coverslips were soaked off of mounted 30 μm frozen sections FB sections. The tissue was processed in floating sections for washing, blocking, application of the primary and secondary antibodies and was remounted in agar.

2.9. Confocal Microscopy for FB Labelled Cell Counting

FB-labeled neurons were visualized using C-Apochromat 10X or 20X objective lenses on a Zeiss LSM510 laser scanning confocal microscope. FB-labeled cell bodies in P1-P3 and S1-S3 segments were identified as having visible nucleus and typical morphology and were counted in each 30 μm cryosection. The unpaired t-test was used determine significant differences in mean value of labelled neuron cell body counts per section in each cord segment.

2.10. Neurofilament Axon Counting

Axons identified by neurofilament staining and the DAB chromogen were counted in transverse paraffin embedded sections at quarter length intervals through the scaffold from the rostral scaffold-cord interface. Axons appeared as discrete dark brown points in cross section within the channel core. Axons were counted using 40x and 100x objective lenses on an Axio Imager Z1 microscope (Carl Zeiss, Inc., Oberkochen, Germany) in the same manner as we have previously described (Chen et al., 2011b; Krych et al., 2009; Madigan et al., 2014). The number of axons in all seven channels at a single quarter length interval section was summated. The three interval sample data points were then averaged to yield a single mean value in each of the 10 animals per group. The unpaired t-test was used determine significant differences in mean axonal counts between the SC and GDNF-SC OPF[+] animal groups.

To determine the spatial orientation of axonal regeneration within the scaffold, a sub-analysis was done to count axons within three ventral channels individually (designated V1, V2 and V3), three dorsal channels (D1, D2 and D3), and the single central channel (C0) across the three scaffold intervals. The rostral-caudal and ventral-dorsal orientation of each harvested spinal cord with its scaffold implant was carefully maintained

in its anatomic position during tissue block paraffin embedding, and care was taken to avoid twisting of the cord around its central axis. Subsequent 8 micron sections through the spinal cord and scaffold implant were cut without changes to the tissue block position and sections were overlaid onto glass slides in a consistent manner. Sections of spinal cord immediately rostral and caudal to the scaffold in each animal were stained with hematoxylin and eosin staining to verify the anatomic orientation of the tissue block. Stained sections of spinal cord tissue were then directly compared and matched to scaffold sections to identify the ventral-most and dorsal-most channels as V2 and D2 respectively, allowing for designation of the remaining channels.

2.11. Unmyelinated and Myelinated Axon Counting

Paraffin-embedded, transverse tissue sections were selected from the halfway point in 10 SC and GDNF-SC OPF+ scaffolds per group, and were stained with antibodies to Tuj-1 and myelin basic protein. Each individual channel was imaged using a Zeiss LSM510 laser scanning confocal microscope at 20x magnification to ensure the entire channel area was captured. The number of axons staining with Tuj-1 alone (unmyelinated axons) or with Tuj-1 co-localizing with MBP (myelinated axons) was estimated using a counting frame fractionator probe within the Stereo Investigator software suite (MBF Bioscience, Williston, Vermont USA, version 11.04). A square grid measuring 100 μm x 100 μm was randomly superimposed over the channel image after the outer channel contour had been accurately circumscribed to define the reference volume. A single counting frame of 50 μm x 50 μm was then generated within the same quadrant of each grid square in sequence, yielding a sampling of 25% of the channel surface area. An axon was counted if it fell either fully within the framed area or lay partially outside but overlapped with the 'acceptance lines'

making up the top horizontal and right hand vertical boundary of the frame. Any axon that overlapped the 'forbidden lines' (bottom horizontal and left-hand vertical boundary), or fell outside of the frame area was not counted. Axon counts were made separately by two blinded observers. Raw counts were recalculated by the software to produce fractional estimator counts for the full channel area. Statistical significance of differences in mean fractional estimates for myelinated and unmyelinated axon counts in 61 SC and 66 GDNF-SC channels was determined by one way ANOVA and Bonferroni's multiple comparisons test. The percentage of myelinated axons in each channel was calculated as the number of myelinated axons divided by the sum of the number of myelinated and unmyelinated axons. The significance of mean differences in the percentage of myelinated axons between the SC and GDNF SC groups was calculated using the unpaired t-test.

2.12. Axon Association with Host Schwann Cells

To determine whether oligodendrocytes or their precursor cells (OPCs) were had migrated into scaffold channels with myelinated axons, scaffold tissue sections from five SC and GDNF-SC implanted animals were at selected from the ¼ and ¾ scaffold length intervals, along with spinal cord tissue sections adjacent to the scaffold. Sections were double-stained with antibodies against the nuclear antigen Olig-2 and MBP, allowing for a survey under confocal microscopy of 29 SC channels 33 GDNF-SC channels. Scaffold sections from the ½ interval length from these same animals were used to double stain with antibodies against P0 and MBP.

To identify an association between regenerating axons and host Schwann cells, SC and GDNF-SC channels from 5 animals per group were immunostained with antibodies to p75, Tuj-1 and myelin basic protein. Each scaffold channel was imaged separately in a series of 3 by 3 image tiles (0.166 um/pixel) by laser scanning confocal microscopy, using a

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40x objective plus an additional optical magnification zoom of 2.5x, and parameters of 10% tile overlap. Full color images were processed into their separate green, red and far red channel tracks. The surface area of each separate color was measured within a standardized image size (254.9 by 254.9 microns) using objection detection stereology software (Neurolucida suite version 11.062, MBF Bioscience). Software settings for object detection sensitivity and size exclusion were kept constant for each color track analyzed. The surface area for each Tuj-1 and p75 object was summated within a single channel and represented a point within a correlation analysis (Figure 6B). The mean total surface area per channel of MBP was normalized to the mean area of Tuj-1 in all channels (n=25 SC and 34 GDNF-SC channels) and compared by the unpaired t-test (Figure 6C).

2.13. Functional Analysis

Hindlimb locomotor function was assessed in open field testing by three independent observers blinded to the animal group at weekly intervals in 25 of 30 SC OPF[+] and 29 of 30 GDNF-SC OPF[+] implanted animals for 4 weeks after surgery. Each animal's movement in an open plastic box was observed for 5 min, and individual ankle, knee and hip joint movement, weight support, toe clearance, tail position, and coordination of paw position placement in gait was recorded. Function was scored by means of the established 21 point Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). The BBB score for each animal was calculated as the average of movement scores between the two hindlimbs, and as the mean score of the three observations. If an animal had an average right or left limb score at week 1 greater than 4 (more than slight movement of all three joints of the hindlimb) it was excluded from further functional analysis. Extensive movements of the hip, knee, and ankle at the week 1 time point was considered to represent an incomplete transection. In our experience, untreated animals with complete

transections do not achieve a score greater than 4 following four weeks of recovery time. A BBB score of 4 or less at week 1 was therefore considered an appropriate inclusion criteria from which to base measurements of subsequent functional improvement.

3.   Results

3.1. SC and GDNF-SC OPF$^+$ Scaffold Implantation

Positively charged oligo (poly(ethylene glycol) fumarate) (OPF$^+$)(Dadsetan et al., 2009) hydrogel scaffolds were fabricated by injection molding and were loaded with wild type Schwann cells (SC) or Schwann cells that were genetically modified with retrovirus to overexpress GDNF and eGFP (GDNF-SC) (Figure S1). Both primary SCs and GDNF-SC released GDNF protein into the culture media over a 24 and 72 hour period *in vitro*. GDNF secretion by the genetically modified cell line was 2-5 x 10^3 fold higher than that from wild type cells. Monolayers of 2.5 x 10^5 primary SCs secreted 3.77 ± 0.69 pg GDNF per ml of assayed culture media (mean $^+$/- SEM) over 24 hours and 4.55 ± 1.28 pg/ml over 72 hours (n=4) (p=0.51). *In vitro* monolayers of 2.5 x 10^5 GDNF-SCs secreted 15,256 ± 594.3 pg GDNF per ml media over 24 hours and 11,869 ± 127.7 pg/ml over 72 hours (n=4). 4,422.0 ± 522.7 pg/ml and 5,833.2 ± 940.4 pg/ml of GDNF was released from OPF$^+$ scaffolds loaded with 2.5 x 10^5 GDNF-SCs *in vitro* at 24 hours and 72 hours respectively (Figure S1). An additive accumulation of growth factor concentration was not seen in either condition between the 1 and 3 day time points, reflecting neurotrophin protein turnover.

Wild type SC and GDNF-SC loaded scaffolds were surgically implanted between the rostral and caudal ends of complete spinal cord transections at the level T9 in 30 animals for each scaffold type. Overall animal mortality was 3/63 animals (4.7%), 3/33 animals (9%) for the SC group, and 0/30 (0%) for the GDNF-SC group. All animals were sacrificed 4 weeks after scaffold implantation. The scaffolds were well aligned and fully integrated between transected ends of the spinal cord on gross tissue pathology. Channels of regenerated tissue extended through the scaffold length.

3.1. Quantification of Axonal Regeneration by Axon Counting

Regeneration into primary SC and GDNF-SC loaded OPF$^+$ scaffolds was quantitated by counting neurofilament stained axons in 10 animals per group, as we have previously described (Chen et al., 2011a; Madigan et al., 2014). The mean number of axons regenerating into GDNF-SC OPF$^+$ polymer scaffolds (2773.0 ± 396.0) was higher than the mean number in primary SC OPF$^+$ scaffolds (1666.0 ± 352.2) ($p<0.05$) (n=10 animals per group) (Figure 1A). A sub-analysis of axons counts was done to determine whether GDNF-SCs supported axonal growth in a particular anatomical region of the scaffold and adjacent spinal cord. Axons were counted in three individual dorsal channels, three individual ventral channels and the central channel. An increase in mean number of centrally-oriented axons (C0) (417.80 ± 85.98) and in the midline ventral channel V2 (661.10 ± 146.10) was observed in the GDNF-SC group (n=10) over C0 (175.60 ± 41.33) (Figure 1B), and V2 (310.70 ± 67.22) in the primary SC group respectively ($p < 0.05$) (Figure 1C). No difference in mean axons counts was observed between SC and GDNF-SC groups in each of the three corresponding dorsal or the two ventrolateral channels.

The channel areas of the OPF$^+$ scaffold were designed to overlap topographically with anatomic ascending and descending tracts in the rat spinal cord (Figure 2). The ventral midline V2 channel would be located over the anterior median fissure, vestibulospinal and rubrospinal descending tracts in the adjacent spinal cord stumps, while the central channel overlaps with descending corticospinal tracts and periaqueductal gray matter.

3.2. Quantification of Fast Blue Retrograde Axonal Tracing

Twenty animals in each of the primary SC and GDNF-SC groups underwent a second surgery three weeks after OPF$^+$ scaffold placement for retrograde axonal tracing to

determine the direction of axon growth through the scaffolds. Fast Blue (FB) dye was injected into the thoracic spinal cord at a distance of five millimeters either rostral to (10 animals) or caudal to (10 animals) the implanted scaffold to label neurons whose axons had respectively ascended or descended through the scaffold and penetrated into the distal cord. Animals were sacrificed for analysis one week after FB injection. Labelled cells were counted in 15 millimeter long segments of spinal cord, designated P1 – P3 rostrally and S1 - S3 caudally. The P1 segment contained the implanted scaffold and FB injection site.

FB labelled neurons had a typical cell body, dendritic and nuclear morphology (Figure 3). Cell-loaded OPF$^+$ scaffolds supported bidirectional, ascending and descending growth of axons through the scaffold and into the distal cord. The mean total number of neuron cell bodies labelled in caudal spinal cord segments by a rostral FB injection (ascending axons originating in segments S1 – S3) was higher (111.80 ± 28.64 per 30 μm longitudinal section) in animals with GDNF-SC OPF$^+$ scaffolds than in animals with primary SC OPF$^+$ scaffolds (12.60 ± 2.60 per section) (p<0.001) (n=10 animals). The mean total number of neuron cell bodies identified in rostral spinal cord segments following caudal FB injection (descending axons originating in segments P1 – P3) was the same in both groups, 19.90 ± 1.68 in SC OPF$^+$ scaffold animals and 19.60 ± 3.42 in GDNF-SC OPF$^+$ scaffold animals (n=10 animals).

When mean FB-labelled cell counts within the individual 15 millimeter segments were compared, the number of labeled cells per group was seen to be evenly distributed through each of the three designated segments and was not observed to significantly decline with distance from the scaffold. Following rostral FB injection, the mean number of labelled neurons counted in caudal cord S1, S2, and S3 sections was 12.00 ± 1.39, 5.11 ± 1.55 and 19.7 ± 5.83 respectively in SC animals (n=10) (p>0.99), and 67.9 ± 27.2, 136.0 ± 52.63 and 117.1 ± 25.67 (n=10) (p>0.36) in GDNF-SC implanted animals respectively. The

mean number of labelled neurons in the rostral cord segments following caudal cord FB injection was 27.70 ± 2.77, 10.00 ± 3.67, and 25.00 ± 3.06 in P1, P2 and P3 sections respectively in SC animals (n=10) (p>0.99), and 26.20 ± 2.27, 12.80 ± 3.83, and 19.70 ± 5.31 respectively in GDNF-SC animals (n=10) (p>0.99).

Tissue segments with FB-labeled neurons were counterstained with antibodies to choline acetyl transferase (CAT), Tuj-1 (Figure 4A) and glial fibrillary acidic protein (GFAP) (Figure 4B). FB fluorescence co-localized with CAT and Tuj-1 antibody staining identifying most of the labelled cells in the caudal S1 section as spinal motor neurons. GFAP positive cells did not co-localize with FB dye, indicating that FB was not taken up by spinal cord astrocytes.

3.3. Regenerating Axon Myelination

The extent to which axons regenerating through SC and GDNF-SC channels began to be myelinated at 4 weeks was analyzed using antibody staining against Tuj-1 and myelin basic protein (MBP) at the ½ scaffold length interval in 10 animals per group. Unmyelinated axons were identified by Tuj-1 staining alone, while the co-localization of Tuj-1 and MBP indicated that the axon was being myelinated in SC and GDNF-SC OPF$^+$ channels (Figure 5A and B). Fractional estimates for the number of unmyelinated and myelinated axons in each SC and GDNF-SC channel were calculated using unbiased stereology. The mean total number of axons (myelinated plus unmyelinated) was increased in GDNF-SC channels (Figure 5C), 243.50 ± 21.27 axons per GDNF-SC channel versus 145.70 ± 13.89 axons per SC channel (p<0.0001)), which confirmed the previous neurofilament axon counting data by a second analytical method.

The majority of regenerating axons in both groups were unmyelinated at 4 weeks post implantation. The mean number of unmyelinated axons per channel was higher in

GDNF-SC channels, 161.50 ± 13.37 axons (n=66 channels, 10 animals) (66.3% of total GDNF-SC axons), than in control SC channels, 112.40 ± 10.74 axons (n=61 channels, 10 animals) (77.1% of total SC axons) (p<0.05). The mean number of myelinated axons was also higher in GDNF-SC channels (81.94 ± 14.75 axons per GDNF-SC channel, 33.7 % of total) than in SC channels, 33.27 ± 5.184 axons, 22.9% of total SC axons (p<0.05)). After controlling for the overall increase in GDNF-SC axon counts, the percentage of axons myelinated remained higher in the GDNF-SC group at 26.07 ± 2.60% (Figure 5D), versus 17.43 ± 1.70% of SC axons (p<0.01), demonstrating a higher efficiency of regenerating axon myelination in the presence of implanted GDNF-SC at 4 weeks.

3.4. Characterization of Axon Myelination by Host Schwann Cells

MBP is present in myelin formed by both peripheral nervous system Schwann cells and central nervous system oligodendrocytes. To determine whether oligodendrocytes or their precursor cells (OPCs) had migrated into OPF+ scaffold channels with myelinated axons, five SC and GDNF-SC scaffolds and adjacent spinal cord sections were double-stained with antibodies against the nuclear antigen Olig-2 and MBP. The adjacent spinal cord was positive for MBP staining in a normal reticular pattern which was interspersed with Olig-2 nuclear staining (Figure 6A). In scaffold channels, MBP staining was consistently observed as characteristic circular rings, while Olig-2 nuclear staining was not observed in any channel at any scaffold interval length (Figure 6B).

To further demonstrate that the myelination within scaffold channels must therefore be derived from Schwann cells, scaffold and adjacent spinal cord sections were double stained with MBP and with myelin protein P0, an antigen that is present only in peripheral nervous system myelin. In the adjacent spinal cord, positive MBP staining alone was observed through the cord parenychma, while MBP and P0 staining co-localized in

thick rings of myelin in a nearby nerve root (Figure 7C). MBP and P0 staining also co-localized in thin myelin rings within scaffold channels (Figure 7D), confirming myelination by Schwann cells in a peripheral pattern within the tissue engineered spinal cord.

To answer the question of whether there was a population of axons which associated with Schwann cells prior to the formation of myelin (a premyelinated state), SC (n=25) and GDNF-SC (n=34) channels were triple immunostained with antibodies to Tuj-1, p75 Neurotrophin Receptor, and MBP. Four qualitative patterns of antibody staining were observed (Figure 7A). 1) Tuj-1+ axons that did not associate with either p75+ cells or MBP were rarely seen. 2) Tuj-1+ axons that directly associated with p75+ cells were consistently seen, and being without MBP staining, were considered to be in a Schwann cell-associated, premyelinated state. 3) Tuj-1+ axons had become bundled into discrete axonal groupings by ensheathing p75+ cells, and were encircled individually by slips of MBP that varied in thickness/maturity. 4) Tuj-1+ axons were circumscribed by thick rings of MBP and no longer associated with p75+ cells in more maturely myelinated channels.

The relationship between axons and p75+ cells was further quantitated by stereology, using object detection software to measure the surface area occupied by Tuj-1 and p75 staining within each channel. p75+ cell density increased in direct proportion to increasing axonal density in SC and GDNF-SC channels (Figure 7B), with strongly positive correlation coefficients (Pearson r=0.8995, p<0.0001 in SC channels, and Pearson r=0.7665, p<0.0001 for GDNF-SC channels). The similarity in the slopes of the relationship, and the increased scaling between animal groups, together suggested that the same underlying process of axon-p75+ cell association seen at 4 weeks was augmented by GDNF-SC implantation. A progression in the extent of MBP expression, normalized to axonal density, was again observed in GDNF-SC channels (Figure 7C) over SC channels.

Surviving implanted Schwann cells expressing eGFP and GDNF were observed by antibody staining in low numbers in GDNF-SC channels at 4 weeks (Figure S2). The mean number of GDNF$^+$ cells observed per channel was 7.74 ± 1.00 cells (n=35 GDNF-SC channels). Cells expressing GDNF were not detected in primary SC channels by immunofluorescence. GDNF$^+$ cells could be seen adjacent to areas of Tuj-1 and MBP staining, but GDNF$^+$ cells were not directly associated with MBP or p75-Tuj-1 complexes (Figure S3). Surface area measurements of GDNF staining did not correlate with axonal density (Pearson r=0.1272, p=0.5356), indicating that the number of GDNF-SCs present at 4 weeks bore no relationship to the number of axons within channels, and that GDNF-SCs must therefore have influenced axonal regeneration and myelination at an earlier time point.

3.4. Functional Analysis

Locomotor function in SC OPF$^+$ (n=25) and GDNF-SC OPF$^+$ (n=29) scaffold animals was scored using the BBB rating scale (Basso et al., 1995) at weekly intervals after surgery. Scores in both animal groups were consistent with complete post-operative paraplegia at weeks 1 and 2 (Figure 8). A progressive improvement in mean hindlimb motor function BBB score was observed in GDNF-SC OPF$^+$ treated animals (2.76 ± .039) over those treated with SC OPF$^+$ scaffolds (1.41 ± 0.34) at week 3 (p<0.05) and at week 4 (3.67 ± 0.40 GDNF-SC and 2.22 ± 0.41 SC, p<0.01).

4. Discussion

4.1. GDNF-SCs Enhanced Axonal Regeneration in OPF[+] Scaffolds over Primary SC Implantation

The engraftment of Schwann cells in the injured spinal cord creates a hybrid peripheral nervous and central nervous system environment which has been widely shown to be among the more effective strategies for repair (Oudega et al., 2005). Schwann cells reduce the size of spinal cord cysts, remyelinate axons and improve functional recovery in animal models of spinal cord injury (Biernaskie et al., 2007). Schwann cells produce a number of growth factors that support the growth of axons, and express cell adhesion molecules on their surface for axon guidance (Tabesh et al., 2009). Autologous human Schwann cells are being used according to current Good Manufacturing Practices (cGMP) in a clinical trial in patients with spinal cord injury (Bunge and Wood, 2012).

The combination of Schwann cell therapies with the delivery of neurotrophic factors further improves regeneration into Schwann cell implants (Xu et al., 1995). Genetically engineered Schwann cell lines (Bo et al., 2011; Liu et al., 1999) have expanded the scope and the efficacy of combination strategies to influence regeneration (Franz et al., 2012). For example, Bunge and colleagues (Kanno et al., 2014) have transplanted Schwann cells genetically modified to secrete both a bifunctional neurotrophin and chondroitinase ABC into a subacute contusion injury in rats. Improvements in the number of myelinated axons, penetration of proprioceptive and corticospinal axons beyond the graft, and recruitment of brainstem neurons through the graft were observed. There were also improvements in locomotor and allodynia sensory function using neurotrophic cells secreting chondroitinase, over that observed with unmodified Schwann cells and single modality cells with the neurotrophin or enzyme alone.

GDNF is a part of the transforming growth factor β signaling family and is closely related to neurturin, artemin and persephin. These ligands preferentially bind to GDNF Family Receptor α subtypes 1 – 4 (GFRα1 - GFRα4) respectively, which signal through Ret tyrosine kinase, glycosyl phosphatidyl inositol, and Src tyrosine kinase subunits (Baloh et al., 2000) to effect gene transcription. GDNF also signals in conjunction with co-receptor coupling to neural cell adhesion molecule (NCAM) on lipid rafts and non-receptor tyrosine kinase Fyn in Ret-negative cell types, including neonatal rat SCs (Iwase et al., 2005). GDNF may require the presence of heparin sulfate proteoglycans for full signaling activation (Barnett et al., 2002).

Several mechanisms of action for GDNF in spinal cord repair after injury have been elucidated in multiple animal models. A direct neuroprotective effect by GDNF on spinal motor neuron survival (Houenou et al., 1996) may relate to meeting the increased trophic requirements of injured cells (Oorschot and McLennan, 1998). The availability of GDNF may inhibit the rate at which neurons default to undergo apoptosis following injury. Injection of GDNF protein into a contusion injury was shown to increase the number of surviving axons and upregulated Bcl-2 expression at the injury site (Cheng et al., 2002). Genetically modified Schwann cells producing GDNF and transplanted into a contusion model were also shown to inhibit neuronal apoptosis via Bcl-2/Bax pathways (Liu et al., 2014). Improved axonal survival likely combines with active axonal extension and sprouting induced by GDNF to contribute to higher number of axon counts within sites of injury. GDNF has been shown to strongly promote spinal motor neuron axonal extension (Bohn, 2004) branching, targeting and synaptic remodeling (Keller-Peck et al., 2001; Markus et al., 2002). Adenoviral delivery of the GDNF gene to the sensorimotor cortex enhanced axonal sprouting of the corticospinal tract following a unilateral lesion at the

level of the pyramids as observed by BDA antegrade tracing (Zhou and Shine, 2003). Similarly, the distance of rubrospinal axonal retraction from a cervical hemisection injury site was shown to be reduced by half by instillation of GDNF-saturated gel foam into the injury (Dolbeare and Houle, 2003). GDNF may favorably impact upon reactive astrogliosis, infiltration of activated macrophages, and cystic cavitation at the graft-host interface (Iannotti et al., 2003). GDNF may influence Schwann cell-astrocyte interactions to reverse the inhibitory properties of the graft-host interface thereby facilitating axon regeneration. Deng *et al.* (Deng et al., 2011) used PAN/PVC guidance channels seeded with SCs either mixed with GDNF protein or SC genetically modified with lentivirus to secrete GDNF and implanted these into a spinal cord hemisection model. GDNF with SCs reduced the proportion of GFAP and CSPG immunostaining at the graft interface and induced a parallel alignment between migrating astrocytes, regenerated axons and myelination.

4.2. GDNF-SCs Enhanced Regenerating Axon Remyelination

The implantation of GDNF-SCs within OPF$^+$ scaffolds improved the number of regenerating unmyelinated axons, myelinated axons and the proportion or the efficiency at which axons became more maturely myelinated at 4 weeks. Others have similarly shown that polymer scaffolds which deliver GDNF and SCs act synergistically to promote the remyelination of regenerating axons. GDNF is known to enhance the migration of endogenous Schwann cells into polymer grafts and improve myelination of regenerating axons (Blesch and Tuszynski, 2003). Iannotti *et al*. (Iannotti et al., 2003) demonstrated that implanting recombinant GDNF with Schwann cells in miniguidance channels had a direct neurite growth promoting effect and enhanced the myelination of ingrowing axons over SCs alone. GDNF alone without SCs promoted the growth of unmyelinated axons. This study was extended to define a six week time course for progressive increases in axon number and myelination

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through poly-acrylonitrile/poly-vinylchloride (PAN/PVC) guidance channels (Zhang et al., 2009).

The majority of axons within OPF⁺ scaffolds remained unmyelinated at 4 weeks in both the SC and GDNF-SC groups. This is a time point when many of the axons would be still developing and therefore would either not be myelinated or might be "pre-myelinated." The presence of MBP in an axon probably reflects more mature myelination, but might also represent pre-myelinated but ensheathed SC-axon units which would already have unregulated MBP expression even though compact myelin may not have been laid down. Our results indicated that a variety of myelination stages or phenotypes within a given channel and across animal groups was present. The myelination phenotypes observed in OPF⁺ channels were strikingly similar to what is known to during the embryonic development of peripheral nerve (Jessen and Mirsky, 2005). By day E14 in rats, Schwann cell precursor cells (SCP) have begun to infiltrate the margins of extending nerves. By E18 irregular groupings of axons and immature Schwann cells have formed into larger ensheathed bundles. Myelination itself begins 3 days later at birth, which involves a complex progression of precursor cell to mature Schwann phenotyping, and "radial sorting" of axon bundles into discrete one cell to one axon by Schwann cell migration, proliferation and cell selection.

Models of tissue engineering in the spinal cord may therefore be facilitating a process of 're-development.' It may be critical to guide the animal back through specific developmental stages rather than to provide more mature tissues by engraftment. The mature Schwann cells we engrafted into OPF⁺ were not remaining in sufficient numbers, and were not participating directly in axonal myelination at 4 weeks. The success of strategies in spinal cord repair may therefore depend how the construct supplies the cues to enable the animal to redevelop. We previously demonstrated (Hakim et al., 2015) that

implanted Schwann cells migrate rapidly out of the OPF[+] scaffold into the adjacent cord where they provide "radial-glia like" strands that may serve as guides for axons and endogenous host Schwann cells to enter the scaffold. This occurred during the first two weeks, followed by rapid disappearance of most of the implanted cells. In the present study we observed a recruitment of endogenous Schwann cell after scaffold implantation, shown as a direct association of p75^{+} cells with axons, a linear relationship with p75 cell and axonal density, and a peripheral nerve pattern of P0/MBP+ myelin in the absence of oligodendrocytes. This process was enhanced by GDNF-SC implantation, and given the expected lifespan of the implanted cell, that influence must have occurred at a relatively early experimental time point to effect early developmental transitions. GDNF is a chemoattractant for SC migration mediated through NCAM/GFR1α co-receptor recruitment and signaling (Paratcha et al., 2003). GDNF is an important signaling cue for Schwann cell proliferation (Hoke et al., 2002), and for the transition of immature p75[+] Schwann cell-axon bundles to mature myelination units, even promoting the myelination of normally unmyelinated small diameter axons (Hoke et al., 2003). The p75 receptor is expressed at high levels in Schwann cells that are in the premyelinating stage, and is upregulated during development and after injury (Cragnolini and Friedman, 2008; Tomita et al., 2007). The binding of other neurotrophins including brain-derived neurotrophic factor (BDNF) to the p75[+] receptor in Schwann cells also plays a key role in developmental myelination (Notterpek, 2003) by activating myelin gene expression (via NF-κB, Oct-6 and Krox-20 transcription factors) and promoting maturation of myelination.

4.3. GDNF-SCs Promoted Ascending Axonal Regeneration

Our previous FB tracing validation study (Chen et al., 2009) in Schwann cell-seeded PLGA scaffolds demonstrated that axonal growth occurred bidirectionally after complete transection, and that axons extended up to 14 mm beyond the scaffold site. Most FB labelled neuron cell bodies whose axons traversed the scaffold length were seen in the ventral horn regions, and were located between 1 – 10 mm of the scaffold-cord interface in both caudal and rostral directions equally. Our current retrograde tracing experiments have shown a similar bidirectional growth pattern of axons in SC channels. Beyond this baseline, GDNF-SCs promoted the robust regeneration of axons which primarily ascended through the scaffold into the rostral spinal cord from distal, caudal neuron cell bodies. The majority of cells labelled by FB tracer could be identified to be spinal motor neurons by colocalization with CAT antibody staining, suggesting that the ascending neuron population attracted by GDNF to grow rostrally through the scaffold may be part of the caudal intraspinal motor neuron circuitry.

The use of antegrade and retrograde tracers has been a key methodology (Tsai et al., 2001) to refine the concept that specific axonal populations regenerate in response to specific neurotrophic signals following spinal cord injury (Lu and Tuszynski, 2008). In work engrafting fibroblasts genetically modified to secrete GDNF into rat spinal cord hemisection and complete transection injuries, Blesch and Tuszynski (Blesch and Tuszynski, 2003) identified that the growth of several caudally and rostrally projecting axon systems into the graft was enhanced by GDNF. Ascending dorsal column sensory axons in the graft were identified by the transganglionic tracer Cholera Toxin B subunit injected into the distal sciatic nerve. Descending propriospinal and brainstem neurons were labelled by retrograde tracers injected into the caudal graft-host interface (FB) and adjacent caudal spinal cord (Diamidino Yellow). Local motor and sensory axon penetration was identified with choline acetyl transferase and calcitonin gene related peptide (CGRP)

antibodies respectively. Antegrade tracer (Biotinylated Dextran Amine) labelled corticospinal axons did not penetrate into the graft.

Other tracing studies have shown that GDNF can promote similar distributions of both descending and ascending regeneration. Following FB injection directly into a PAN/PVC graft seeded with Schwann cells and recombinant GDNF, increased labelling of bidirectional motor interneurons and descending propriospinal neurons was observed (Iannotti et al., 2003). Recombinant GDNF delivered intrathecally by a miniosmotic pump improved axonal sparing of descending propriospinal, reticulospinal and vestibulospinal neurons after contusion injury as measured by the number of neurons labelled by FluoroGold (FG) retrograde tracing(Iannotti et al., 2004). Xu's group (Deng et al., 2013) have also recently shown that descending propriospinal neurons regenerated across a hemisection lesion into the distal host cord in response to the implantation of GDNF-secreting Schwann cells within a PAN/PVC miniguidance channel. Descending propriospinal neurons were shown to form synapses with host neurons, were efficiently remyelinated by implanted GDNF-secreting cells, and contributed to partial restoration of electrophysiologic and locomotor recovery. In another study, serotinergic fibers were observed to descend through a sciatic nerve graft in the presence of GDNF following complete spinal cord transection (Guzen et al., 2009).

Adenoviral delivery of GDNF injected two mm rostral to a spinal cord contusion injury improved locomotor recovery and increased the number of CGRP positive neurons in the dorsal cord injury site, suggesting that the rescue of ascending sensory neurons may be important for functional outcome (Tai et al., 2003). Intrathecal infusion of GDNF led to ascending regeneration of large fiber (NF200$^+$) and small fiber CGRP$^+$ and purinoceptor P2X$_3$$^+$ sensory axon populations through dorsal root entry zone (DREZ) and into the spinal cord after root crush injury, improving thermal and mechanical nociception, grid walking

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and nerve electrophysiologic function (Ramer et al., 2000; Smith et al., 2012). Small caliber $P2X_3$ neurons express GFRα1 and Ret tyrosine kinase receptor units and may be selectively responsive to GDNF (Bradbury et al., 1998), while larger caliber sensory fibers express both GFRα1 and Ret receptor complexes in addition to other neurotrophic factor receptors including trkB and trkC for high affinity BDNF and NT-3 signaling respectively (McMahon et al., 1994). Several studies of note have shown also that Artemin, a ligand in the GDNF family that binds the GFRα3 receptor subtype, also promotes sensory neuron regeneration across through the DREZ and into the spinal cord after injury (Wang et al., 2008), and restores discrete projections of myelinated muscle, cutaneous and unmyelinated nociceptive afferent populations to specific lamina topographies (Harvey et al., 2010).

4.4 GDNF-SCs in OPF⁺ Scaffolds Promoted Partial Functional Locomotor Recovery Following Complete Spinal Cord Transection

GDNF-SC animals demonstrated significantly improved locomotor function at 3 and 4 weeks post injury compared to animals in the SC group. A delayed, but progressive rate of recovery at weeks 3 and 4 would be more consistent with the development of regenerated tissue as a mechanism for recovery than with neuroprotection and collateral sprouting of preserved tissue at early timepoints. Several factors have likely contributed to the partial recovery of locomotor function observed in our completely transected animals. By implanting neurotrophic factor-secreting Schwann cells within a permissive hydrogel substrate, a critical threshold for total axon number within scaffolds may now support sufficient motor reinnervation across the transected spinal cord segments. The number of axons regenerating after GDNF-SC OPF⁺ implantation exceeds what we have been able to produce with unmodified Schwann cells or any other cell type we have tried in this model.

The proportion of regenerating axons that were myelinated was also increased by GDNF-SC implantation, which may have improved physiologic efficiency of signal conduction. The spatial distribution of axon populations within scaffolds was found to be more concentrated in the central and ventral midline channels loaded with GDNF-SC. The orientation of these central and ventral midline channels was designed to correspond with the location of caudally projecting tracts in the rat spinal cord. When we observed a migration of transplanted Schwann cells from within the channels of the OPF+ scaffolds, this occurred differentially at higher densities out into the caudal spinal cord than into rostral cord during the first 2-3 weeks after transplantation (Hakim et al., 2015). The efflux of transplanted SC into the caudal cord was maximal at Day 14. GDNF expressing SCs transplanted in this study may similarly have migrated into the caudal cord and produced a sufficient amount of GDNF to modulate descending central and ventral midline motor circuits. GDNF has been shown to enhance neuronal plasticity and the expression of synaptic markers such as synaptophysin both *in vitro* and after SCI *in vivo* (Koelsch et al., 2010). GDNF signaling also plays a direct role in presynaptic differerentiation, through vesicle size, clustering, recycling, and to promote precise synaptogenesis in developing neurons (Ledda et al., 2007; Paratcha and Ledda, 2008).

Changes in caudal synaptic plasticity and descending input due to GDNF-SC migration may combine with longer distance intraspinal motor neuron connections made by ascending axons to enhance central pattern generator reprograming. The regeneration of rostrally projecting, ventral CAT+ motor interneurons identified by retrograde tracer analysis in this study may specifically be important to recovery. Axonal regeneration through a graft and deep into the distal cord is established to be essential for functional neurologic recovery. Functional recovery has not been observed in several studies that demonstrate robust axonal regeneration into grafts but in which the axons failed to emerge

from the graft into the distal cord (Blesch and Tuszynski, 2009). In this study, we demonstrate regeneration of axons not only into the implanted scaffolds, but at least 5 mm into the distal spinal cord both rostrally and caudally. These bridging axons may form functional connections across the lesion site.

Endogenous GDNF production in the rostral spinal cord following a complete transection injury could provide an early gradient cue for ascending axonal regeneration. GDNF mRNA and protein expression is upregulated in neurons in the rostral stump ventral horn grey matter (Zhou et al., 2008) at an earlier time point (3-7 days) than in the caudal stump (day 14). GDNF expression by transplanted SC in our study could have improved ascending axonal regeneration by supplementing the bias towards a higher concentration gradient of GDNF within the rostral cord stump. It has also been previously demonstrated that ascending axons are better able to regenerate through inhibitory factors within the lesion site such as myelin breakdown products and chondroitin sulfate proteoglycans (Blesch and Tuszynski, 2009) than descending axons. Ascending neuronal projections may help promote functional recovery by restoring neuronal relays as bridges across the lesion site. Rebuilding neuronal relay circuits using transplanted neural stem cells or fetal grafts has been shown to be effective in improving functional recovery after SCI (Bonner and Steward, 2015). Remodelling in lumbosacral intraspinal motor circuity has been hypothesized to contribute to the ability of neonatal rats to recover locomotor function after complete spinal cord transection, given that these animals can recover function without any evidence of axonal regeneration through the proximal injury site and the caudal cord is isolated from descending inputs (Tillakaratne et al., 2010). Furthermore, ascending inputs from propriospinal neurons that synapse on spinal circuits to integrate descending and ascending inputs are important for motor planning and evaluation (Hantman and Jessell, 2010). Restoration of these inputs through the enhanced ascending

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axon regeneration with GDNF-SC transplantation could play an important role in the improved functional recovery observed in these animals. Weight-bearing movement, which is controlled by sensorimotor circuits integrated into the lumbosacral spinal cord, can be reinforced by repetitive task learning and epidural stimulation, despite complete disruption of supraspinal input (Edgerton and Roy, 2009) following injury. The activity-dependent adaptation that leads to functional recovery of stepping control in rats, cats and humans relies essentially on the reorganization of ascending relay connections for processing proprioceptive information (Courtine et al., 2008).

4.5 Translational Implications

An FDA-approved, multicenter clinical trial (NCT02138110, University of Arizona) is underway to evaluate whether poly(lactic-co-glycolic acid) (PLGA) scaffold implantation is safe as a treatment for complete spinal cord injury in humans. This pioneering study will set important precedent for the feasibility of new applications for Investigational Drug (IND), Investigational Device Exception (IDE) and clinical trial approval for the use of polymers like OPF[+]. We have recently published a review (Staff et al., 2014) analyzing the new initiatives from U.S. federal regulatory and funding agencies which aim to accelerate the process of clinical translation. The main barriers to successful clinical translation have been identified at the transition or hand-off of a technology between academia and industry, and during preclinical transfer of manufacturing from the research laboratory to a cGMP facility (NIH, 2007) . Regulatory issues in early stage clinical trials may cause difficulties given a lack of knowledge about the regulatory approval process among academic scientists (Angius et al., 2012). Meanwhile, scientists may expect that data publication is their *raison d'être*, and beyond this, a biomedical or pharmaceutical company should or will be the entity that comes in and develops a translational product.

While most approaches using spinal cord polymer implants aim to discover disease-specific therapies, few studies are designed to successfully develop a new patient therapy. A critical principle is that researchers learn about the process of translation, and use their knowledge to inform the design of experiments at the outset of a project so that data will be suitable for use in future submissions to the FDA. As to the future translation of GDNF-SC OPF$^+$ scaffolds, large animal studies will need to be designed in such a way that all of the individual components of scaffold-cell-molecule model are suitable for human use. It is essential that cGMP manufacturing is optimized prior to use in the large animal, so that the final polymer put into the animal will be the same as will be used for human clinical trials. The Schwann cells will be derived as an autologous human transplant. Gene modifications for cell-based molecular therapy will require that the our retroviral expression vector be changed to either to a non-integrating vector, or to an integrative approach that minimizes insertional mutagenesis risk, as may be accomplished for example using custom nucleases targeted to genomic safe harbor sites. 'Freedom to operate,' or the legal rights to use each component of the proposed model as intended during a trial, will need to be clearly established, by seeking out licensing agreements with current patent holders or establishing *de novo* intellectual property prior to embarking on large animal studies. Institutional and administrative expertise is needed to facilitate complex negotiations between the clinician-investigator and regulatory agencies, funding sources, and commercial suppliers. The investigator team must also foster close, collaborative relationships with personnel who will be essential to actually conducting the clinical trial, neurosurgical specialists, inpatient unit coordinators, and study trial coordinating staff. The role of the clinical investigator therefore, ultimately, is to be proactive in the production of scientific data and institutional infrastructure that supports the current framework of accelerated initiatives.

5.1. Conclusions

OPF+ scaffolds delivering GDNF-SC promoted significantly higher numbers of axons within the scaffold than primary SCs, which had represented our best cell type to date. There were increased numbers of axons within central and ventral midline channels of GDNF-SC scaffolds compared to SC scaffolds. The number of axons ascending from caudal intraspinal motor neurons through OPF+ scaffolds was ten-fold higher in the GDNF-SC group. The implantation of GDNF-SCs enhanced the myelination of regenerating axons by host animal Schwann cells. Phenotypic patterns of premyelination, axon bundling, and mature myelination in OPF+ scaffolds are similar to those seen in embryonic peripheral nerve development. Oligodendroctyes were not seen within scaffold channels. Completely transected animals implanted with GDNF-SC OPF+ scaffolds recovered partial locomotor function. This represents the first time we have observed significant recovery following severe spinal cord injury using our model of combination therapy. Future clinical translation of the GDNF-SC OPF+ model will depend next upon establishing cGMP polymer production, autologous human Schwann cell cultures, expression vector safety modification and 'freedom to operate' in preparation for a large animal study.

Mr. James Tarara at Mayo Clinic for his expertise in confocal microscopy imaging; Mr. Trent Chiang for his technical assistance with the GDNF ELISA studies.

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