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PRINCIPAL INVESTIGATOR: Treena Arinzeh

CONTRACTING ORGANIZATION: New Jersey Institute of Technology
Newark, NJ 07102

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Treena Arinzeh, Mary Bunge, Mesut Sahin, Yee-Shuan Lee, Siliang Wu, Sinan Gok

E-Mail: arinzeh@njit.edu

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New Jersey Institute of Technology
323 Martin Luther King Jr Blvd
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14. ABSTRACT

This study proposes a novel tissue engineering strategy to promote functional recovery after spinal cord injury. The approach utilizes piezoelectric conduits, neurotrophins and Schwann cells (SCs) to promote axonal regeneration. Piezoelectric materials provide electrical stimulation in response to minute mechanical deformation. By providing a strategy that can stimulate regeneration through physical as well as biochemical cues, axonal regeneration can be achieved into, through and out of conduit as well as into the caudal cord to improve functional recovery. This project is (1) evaluating different designs of piezoelectric conduits with SCs with or without Matrigel and (2) determining if axons extend into the caudal spinal cord parenchyma following the controlled release of neurotrophins at and below the SC conduit. Findings to date demonstrate that in the rat complete transection model, axons extend along the length of the conduit when the conduit is filled with aligned fibers and improved SC survival is achieved using this design when SCs are combined with Matrigel. The fibrous layers consisting of aligned fibers that fill the conduit enable additional contact guidance for directed axon regeneration along the rostral/caudal axis.

15. SUBJECT TERMS

Polyvinylidenefluoride-trifluoroethylene (PVDF-TrFE), piezoelectric, aligned, electrospun, spinal cord repair, Schwann cells, Matrigel, brain-derived growth factor (BDNF), neurotrophin-3 (NT3), Plurronics, controlled release, functional recovery.

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1. INTRODUCTION

This proposal seeks to improve axonal regeneration and functional recovery in spinal cord injury (SCI) by utilizing a piezoelectric conduit with SCs that provides both physical and neurotrophic cues in combination with neurotrophin release caudal to the injury. The design of the piezoelectric conduit that is most efficacious in promoting axonal regeneration will be determined. The conduit design will be further developed to achieve controlled release of two neurotrophins and also neurotrophin delivery caudal to the injury to promote axonal regeneration into, through and out of the SC conduit and into the caudal cord to improve functional recovery.

2. KEYWORDS

Polyvinylidenefluoride-trifluoroethylene (PVDF-TrFE), piezoelectric, aligned, electrospun, spinal cord repair, Schwann cells, Matrigel, brain-derived growth factor (BDNF), neurotrophin-3 (NT3), Pluronics, controlled release, functional recovery.

3. ACCOMPLISHMENTS

What were the major goals of the project?

1. Evaluate the design of piezoelectric conduits filled with aligned fibrous layers and SCs with or without Matrigel for axonal growth.
2. Determine if axons extend into the caudal spinal parenchyma following the controlled release of neurotrophins at and below the SC conduit to improve exit of regenerated axons from the conduit and improve functional recovery.

What was accomplished under these goals?

1. Evaluate piezoelectric conduits with SCs for axonal growth with or without Matrigel.

a. Acuro Approval – Period: 0 months, 100% completed
b. Fiber fabrication, characterization, and *in vitro and in vivo* piezoelectric characterization, Period: 1-12 months, 90% completed
c. *In vitro* characterization of Schwann cell survival, myelination and neurite extension on piezoelectric fibers, Period: 1-12 months, 100% completed
d. Piezoelectric conduits will be evaluated in a complete transection model, Period:1-12 months, 100% completed

2. Determine if axons extend into the caudal spinal parenchyma following the controlled release of neurotrophins at and below the SC conduit and improve functional recovery.

a. *In vitro* characterization of the controlled release of NT3 and BDNF from the fibrous piezoelectric scaffolds will be evaluated for neurite extension and SC survival, Period: 13-24 months, 80% completed.
b. *In vitro* characterization of the controlled release of NT3 and BDNF from the PEO hydrogel will be evaluated and DRG neurite outgrowth measured, Period: 13-24 months, 30% completed
c. A pilot *in vivo* study will be performed to determine axon growth into, through and out of the SC-piezoelectric conduit with NT3 and BDNF release, Period: 19-24 months, 50% completed.
d. The full in vivo study will be performed to determine axon growth into, through and out of the SC- piezoelectric conduit with NT3 and BDNF release, Period: 25-36 months, 0% completed.

1. **Major Activities (Period 13-24 months)**
   The major activities accomplished during this period were:
   a. *In vitro* characterization of controlled release of a model protein from piezoelectric scaffolds to determine optimal formulation for NT3 and BDNF.
   b. *In vitro* studies characterizing neurotrophin release from the scaffolds were conducted.
   c. Cytocompatibility studies of the piezoelectric scaffold formulation for controlled release.
   d. *In vitro* studies of SC myelination of axons on scaffolds were conducted.
   e. The co-culture using purified DRGs with SCs was optimized for neurotrophin release studies.
   f. The *in vitro* studies were performed to develop the hydrogel for neurotrophin release caudal to the transplantation site.
   g. *In vivo* studies evaluating the influence of Matrigel in conduits filled with aligned fibers was completed.
   h. *In vivo* studies using piezoelectric conduits filled with aligned fibers having an optimal spacing or hollow conduits containing SCs were performed and results analyzed in the rat complete transection model.
   i. The pilot *in vivo* study using piezoelectric conduits filled with layers of aligned fibers that release neurotrophins was performed.

2. **Specific Objectives (Period 13-24 months)**
   The specific objectives were as follows:
   a. The model protein, lysozyme, was characterized for controlled release from the PVDF-TrFE scaffold formulation.
   b. Neurotrophin release was characterized from the PVDF-TrFE scaffold formulation *in vitro* to establish the release kinetics prior to in vitro cell studies.
   c. The studies for establishing SC growth on the PVDF-TrFE scaffold formulation for controlled release were established and repeated to ensure reproducibility.
   d. SC myelination of axons of dissociated neurons from DRGs on PVDF-TrFE scaffolds was performed *in vitro*.
   e. Methods for the co-culture of purified DRGs with SCs on scaffolds were optimized prior to *in vitro* cell studies of neurotrophin release.
   f. The hydrogel formulation for neurotrophin release caudal to the implantation site was examined *in vitro*.
   g. Findings from *in vivo* studies examining SCs and the use of matrigel loading in the conduit in the complete transection model in the rat were analyzed.
   h. Piezoelectric conduits, either as hollow tubes or filled with aligned fibers having an optimal spacing, containing SCs were implanted in the complete transection model in the rat and results analyzed.
   i. Pilot study examining conduits having neurotrophin release was conducted and results analyzed.

3. **Significant Results or Key Outcomes:**

Major Findings

***In vitro* lysozyme release study.** In preparation for the *in vitro* characterization of controlled release of neurotrophins, NT3 and BDNF, PVDF-TrFE scaffolds were modified and evaluated for the controlled release of protein using lysozyme, which is a model protein having similar molecular weight and charge as the neurotrophins. Polyethylene oxide (PEO) was added to PVDF-TrFE to act as a carrier for the protein. Lysozyme and PEO were dissolved in DI water

and mixed to form a homogenous solution. PEO/Lysozyme solution was added to 20% PVDF-TrFE solution and mixed for half hour followed by sonicating for 2 minutes prior to electrospinning. Two scaffolds groups were evaluated: 1) PVDF-TrFE/PEO/Lysozyme (PPL) with composition ratio of 100:1:0.5; 2) PVDF-TrFE/PEO/Lysozyme/Sucrose (PPLS) with composition ratio of 100:1:0.5:5. Sucrose was used as a second additive to accelerate protein release from the fibers. Sucrose has been shown to be effective in other drug delivery systems [1, 2]. Electrospun scaffolds were cut into disks and immersed in PBS to evaluate lysozyme release from the scaffolds. PBS was collected and replenished over time and the amount of lysozyme was quantified using a lysozyme assay (Fisher Scientific). Sustained lysozyme release was achieved by adding sucrose to the polymer fibers as shown in Figure 1.

[1] Maeda H, Brandon M, Sano A. Design of controlled-release formulation for ivermectin using silicone. International Journal of Pharmaceutics. 2003;261:9-19.
[2] Maeda H, Ohashi E, Sano A, Kawasaki H, Kurosaki Y. Investigation of the release behavior of a covered-rod-type formulation using silicone. Journal of Controlled Release. 2003;90:59-70.

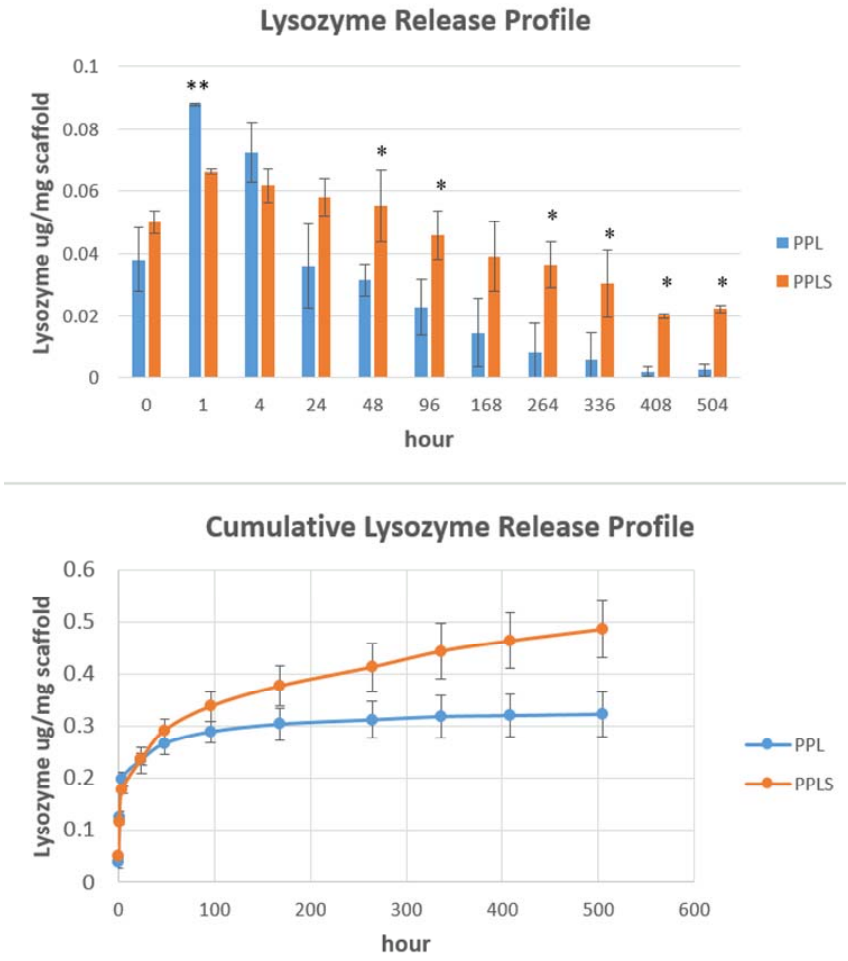

Figure 1. Lysozyme release profile. *Lysozyme release was significantly higher for PPLS (PVDFL-TrFE with PEO and Sucrose) than for PPL (PPVDF-TrFE with PEO). **Lysozyme release was significantly higher for PPL than PPLS.

***In vitro* study of controlled release of neurotrophins from scaffolds.** As demonstrated above, the model protein lysozyme showed that controlled release could achieved by adding sucrose to the PVDF-TrFE scaffolds containing polyethylene oxide (PEO). Sucrose incorporated scaffolds supported SC proliferation and DRG neurite extension (see next section). Thus, sucrose

incorporated scaffolds were used for all neurotrophin release studies. We also investigated the effect of UV sterilization on neurotrophin release kinetics. The release of BDNF and NT3 from the scaffold over time is shown in Figure 2. The scaffolds were not subjected to UV sterilization. A burst release was observed during the first day followed by a sustained/controlled release over the 3 week period. For *in vitro* cell studies and *in vivo* studies, we typically use a UV sterilization time of 20 min per side of the scaffold. As shown in Figure 3, the amount of BDNF and NT3 released decreased to negligible levels when using UV sterilization for 20 min on each side as compared to unsterilized scaffolds. We evaluated the effect of reducing UV sterilization time to 5 min and 15 min on each side of the scaffold on neurotrophin release. For 15 min sterilization, the amount of BDNF and NT3 released decreased to undetectable levels (data not shown). For 5 min sterilization, the amount of BDNF and NT3 released was almost comparable to unsterilized scaffolds (Figure 4). Based on these findings, we are currently performing cell culture studies using the 5 min UV sterilization protocol.

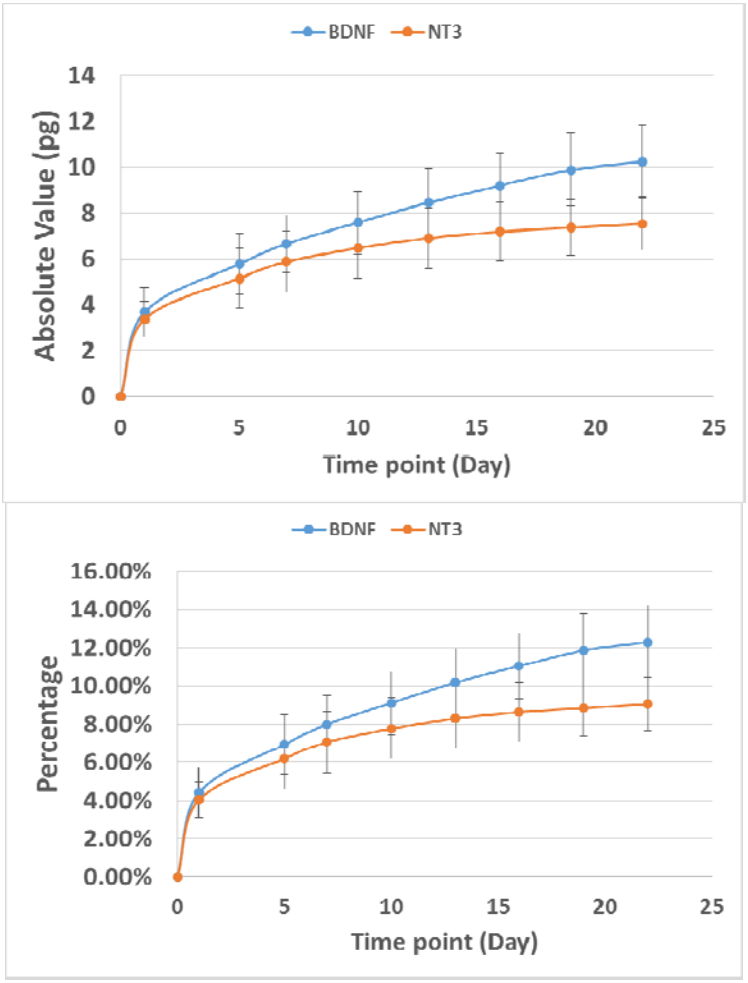

Figure 2. Cumulative release of neurotrophins BDNF and NT3 from scaffolds (unsterilized). Absolute value/amount in pg (Top) and percentage of total amount loaded into the scaffold (bottom).

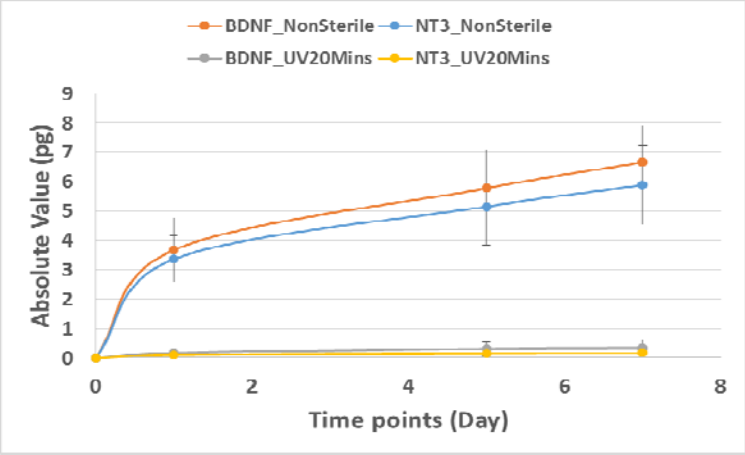

Figure 3. Cumulative BDNF and NT3 release from scaffolds with UV sterilization for 20 min per side of scaffold or without UV sterilization.

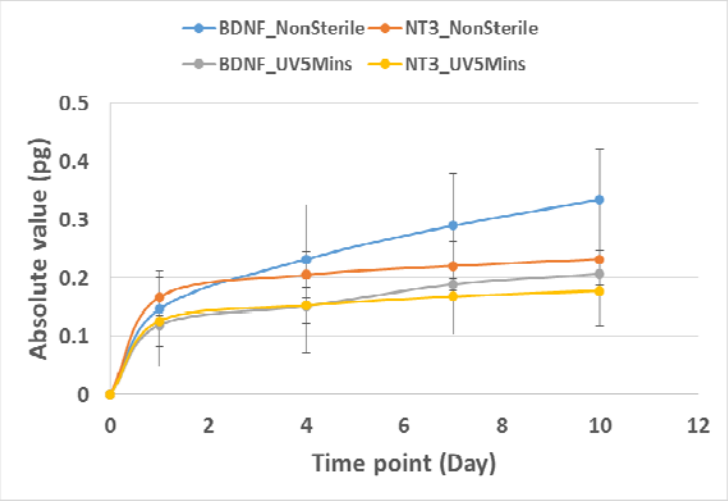

Figure 4. Cumulative BDNF and NT3 release from scaffolds with UV sterilization for 5 min per side or without sterilization.

Cytocompatibility studies of the piezoelectric scaffold formulation for controlled release. Rat SCs, isolated from adult sciatic nerve and transfected to express GFP, provided by the Bunge Lab, were cultured on PVDF-TrFE/PEO and PVDF-TrFE/PEO/Sucrose scaffolds, where the PEO and sucrose additives are being used for the neurotrophin delivery, for up to 14 days using standard growth media for SCs to determine if the scaffold formulation with PEO and sucrose has any adverse effects on cell attachment and growth. Both experimental groups were coated with Matrigel. Tissue culture polystyrene coated with Matrigel was used as a control. SC growth was assessed by changes in cell number using the Picogreen assay over time at 1, 4, 7, 10 and 14 days. SCs seeded on scaffolds continued to grow over time for both scaffold groups (Figure 5). Cell morphology and attachment was assessed by confocal microscopy. As shown in Figure 6, SCs attached well to both groups.

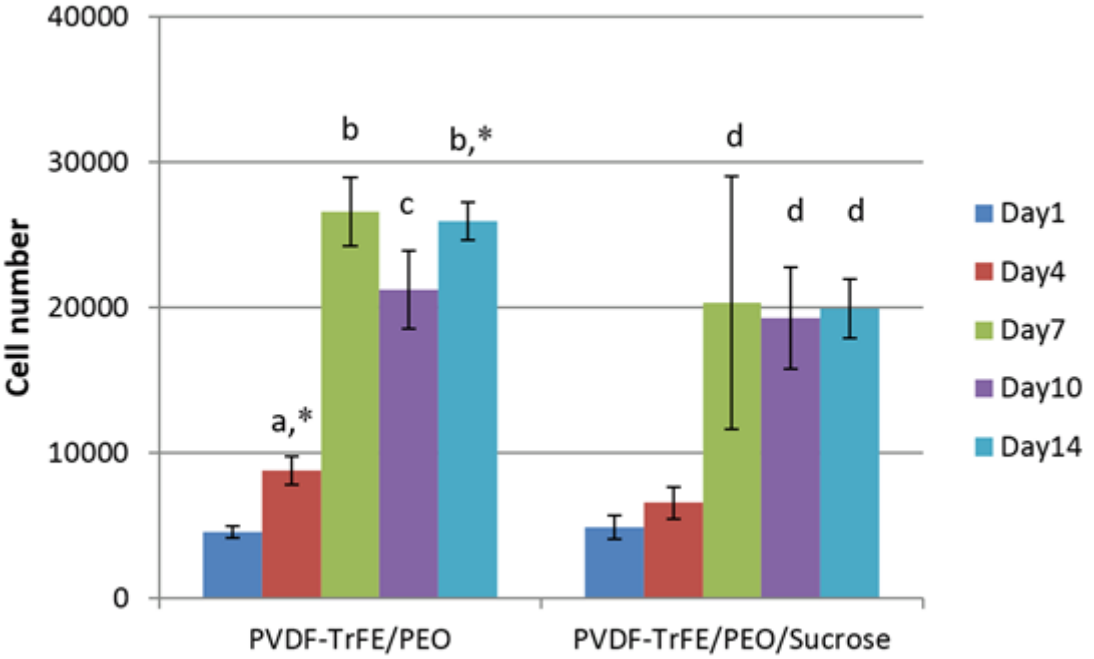

Figure 5. SCs grown on PVDF-TrFE/PEO and PVDF-TrFE/PEO/Sucrose scaffolds for up to 14 days. [a]$p<0.05$, day 4 is significantly higher than day 1, [b]$p<0.05$, days 7 and 14 are significantly higher than other time points, [c]$p<0.05$, day 10 is significantly higher than days 1 and 4, [d]$p<0.05$, days 7, 10 and 14 are significantly higher than days 1 and 4. [*]$p<0.05$, cell number on PVDF-TrFE/PEO scaffold is significantly higher than PVDF-TrFE/PEO/Sucrose scaffold at days 4 and 14.

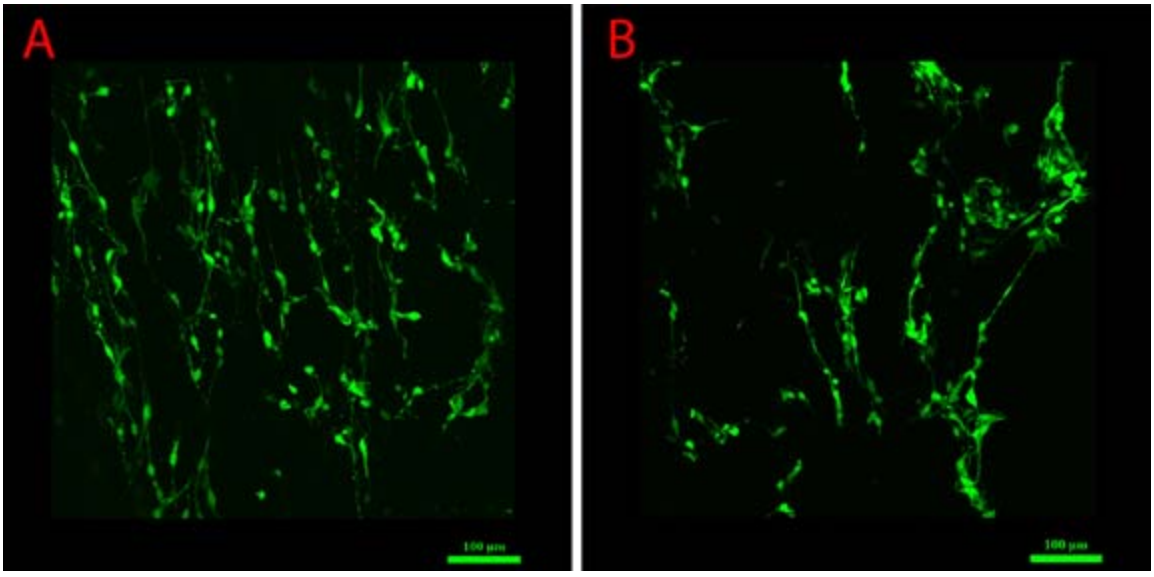

Figure 6. Confocal microscope images of GFP-SCs seeded on A) PVDF-TrFE/PEO scaffold and B) PVDF-TrFE/PEO/Sucrose scaffold at day 10. (Mag 20X, scale bar = 100 um)

Since sucrose incorporated scaffolds will be used for all neurotrophin release studies in year 2, we further evaluated the cytocompatibility of sucrose. We repeated the same study using scaffolds with PEO and low (1%) and high (5%) concentrations of sucrose. Similar SC proliferation results were observed for all three groups (Figure 7).

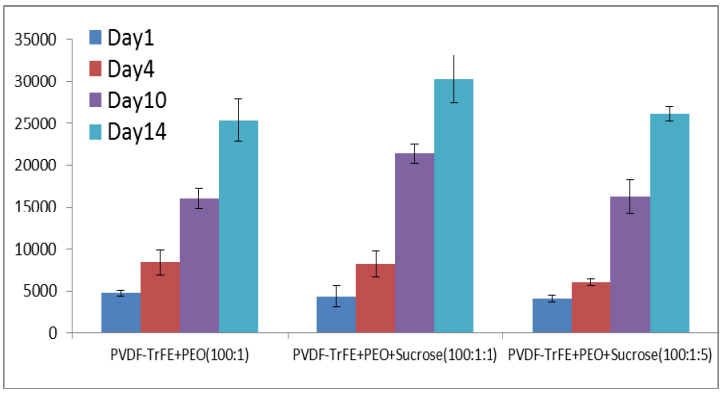

Figure 7. SC number on PVDF-TrFE scaffolds containing PEO with low and high concentrations of sucrose. *SC number on 1% sucrose group is significantly higher than other two groups at day 10. ** SC number on 1% sucrose group is significant higher than 0% sucrose group at day 14.

In addition, SCs were cultured on Matrigel coated tissue culture plastic in media containing sucrose at various concentrations to determine the effect of sucrose on SC growth. The results (Figure 8) demonstrate that sucrose does not affect SC proliferation.

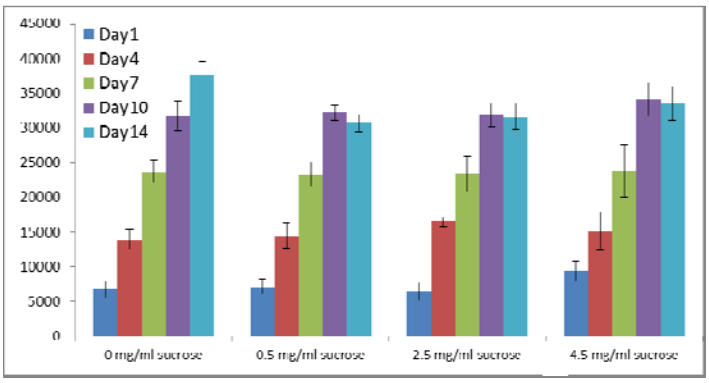

Figure 8. SC number on Matrigel coated tissue culture polystyrene with varying amounts of sucrose added to the media. *SC number in 4.5 mg/ml sucrose media is significantly higher than 2.5 mg/ml group at day 1. ** SC number in 0 mg/ml sucrose media is significantly higher than 0.5 and 2.5 mg/ml groups at day 14.

DRGs isolated from E17 rat embryos were seeded on the Matrigel coated PVDF-TrFE/PEO and PVDF-TrFE/PEO/Sucrose scaffolds and the culture was maintained for 2 days. The culture was fixed using 4% paraformaldehyde and stained for neurofilament. Neurite extension was assessed by measuring 10 longest axons for each DRG. DRGs extended neurites on both scaffold groups and the average neurite extension was 3187.94 ± 265.82 μm on PVDF-TrFE/PEO scaffold and 3273.73 ± 244.15 μm on PVDF-TrFE/PEO/Sucrose scaffold (Figure 9).

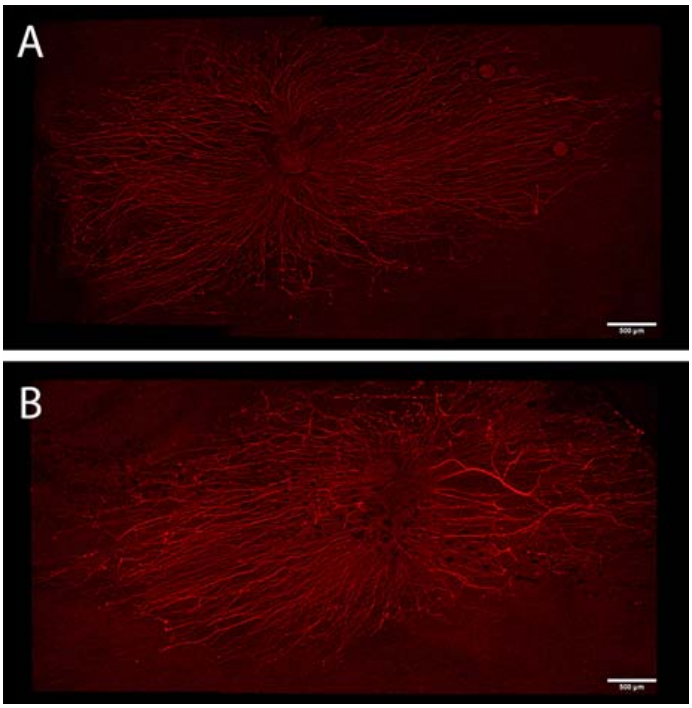

Figure 9. Rat embryo DRG neurite extension on A) PVDF-TrFE/PEO scaffold and B) PVDF-TrFE/PEO/Sucrose scaffold. DRGs are stained with neurofilament (Red) at day 2. (Mag 4X, scale bar=500 μm)

Overall, the findings look favorable demonstrating that the PVDF-TrFE/PEO/Sucrose formulation will support cell growth and neurite extension. Based on these promising findings showing controlled release and adequate cell growth and neurite extension, in vitro studies examining the release of the neurotrophins from the PVDF-TrFE/PEO/Sucrose in SC and DRG co-cultures will be pursued in the next quarter.

SC myelination of axons on PVDF-TrFE scaffolds *in vitro*. To establish SCs on PVDF-TrFE fibrous scaffolds myelinate axons, we performed studies using dissociated neurons from rat dorsal root ganglia (DRG). The PVDF-TrFE scaffolds and glass coverslips, which served as a positive control substrate, were coated with Matrigel overnight. Neurons were seeded onto the substrates and were cultured in neurobasal media with anti-mitotic agents for 15 days to remove any endogenous dividing cells (e.g. SCs and fibroblasts). Purified SCs, at 150,000 cells per culture, were added to the neuron containing culture and maintained for 3 days in fetal bovine serum (FBS)-containing media. Media was changed to myelinating media (FBS-media with 50 μg/ml ascorbic acid) and cultured for 12 days, changing media every two days. The cultures were fixed and immunostained for neurofilament (NF) and myelin basic protein (MBP). Samples were viewed using a standard epi-fluorescent microscope. Myelination, as indicated by the MBP stain along the length of the axon, could be detected for SCs in co-culture with neurons on PVDF-TrFE scaffolds (Figure 10).

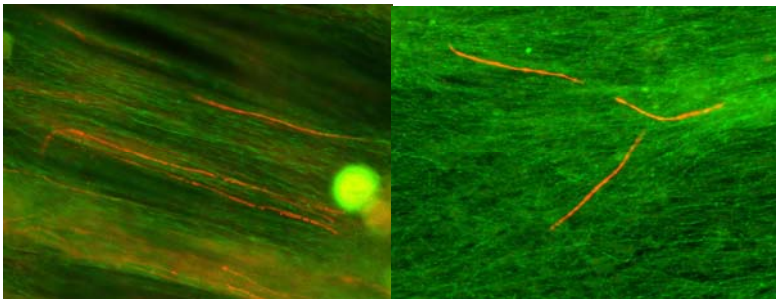

Figure 10. SC-neuron co-culture on PVDF-TrFE scaffolds (A) and glass coverslips (positive control) (B). NF (green) and MBP (red) immunostaining. Myelination, as indicated by the MBP staining, can be seen along the length of the axons on both substrates. (20x obj.)

Purified DRG explant co-culture with SCs on PVDF-TrFE scaffolds was optimized in preparation for neurotrophin release cell culture studies. We have previously established DRG explant/Schwann cells coculture using unpurified DRG explants which contains endogenous Schwann cells and fibroblasts. In order to eliminate the effects that these endogenous cells could have on DRG neuron behavior, we developed and optimized the co-culture using purified DRG explants. Lumbar DRG explants isolated from E17 rat embryos were seeded on collagen coated petri dishes in purification media for one week followed by culturing in maintenance media for additional three days. Maintenance media was changed every day. On the day of seeding DRG explants, axons regenerated from the DRGs body were carefully cut and the DRG bodies were lifted up and seeded onto PVDF-TrFE scaffolds preloaded with SCs. In our previous studies, unpurified DRGs extended neurites in D10 media with or without the presence of NGF. When purified DRGs were used, neurons did not extend neurites in media without NGF after one week in culture (Figure 11). When NGF is added to the media, neurons extended long neurites at 1 week (Figure 12). Future studies examining neurotrophin release in co-culture will use this formulation of adding NGF to the media.

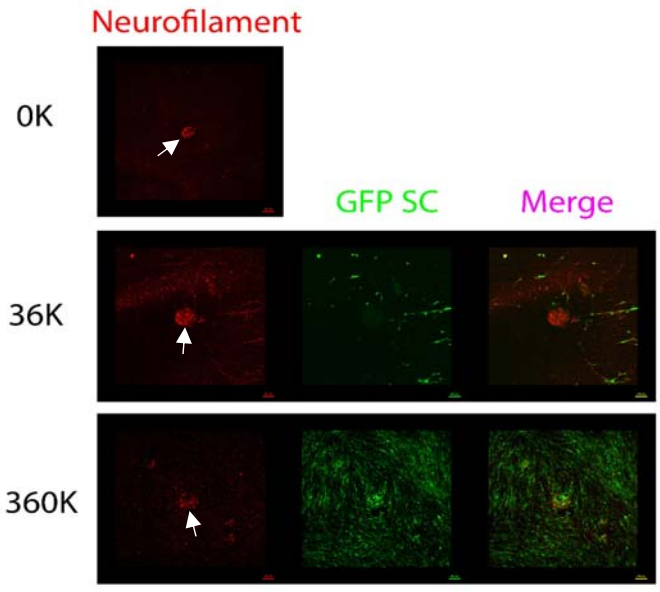

Figure 11. Purified DRG explants (white arrow and stained red) cultured in media with BDNF and NT3, but without NGF at 1 week. SC (stained green) seeding density 0K, 36K, 360K. (10X, scale bar = 100 μm).

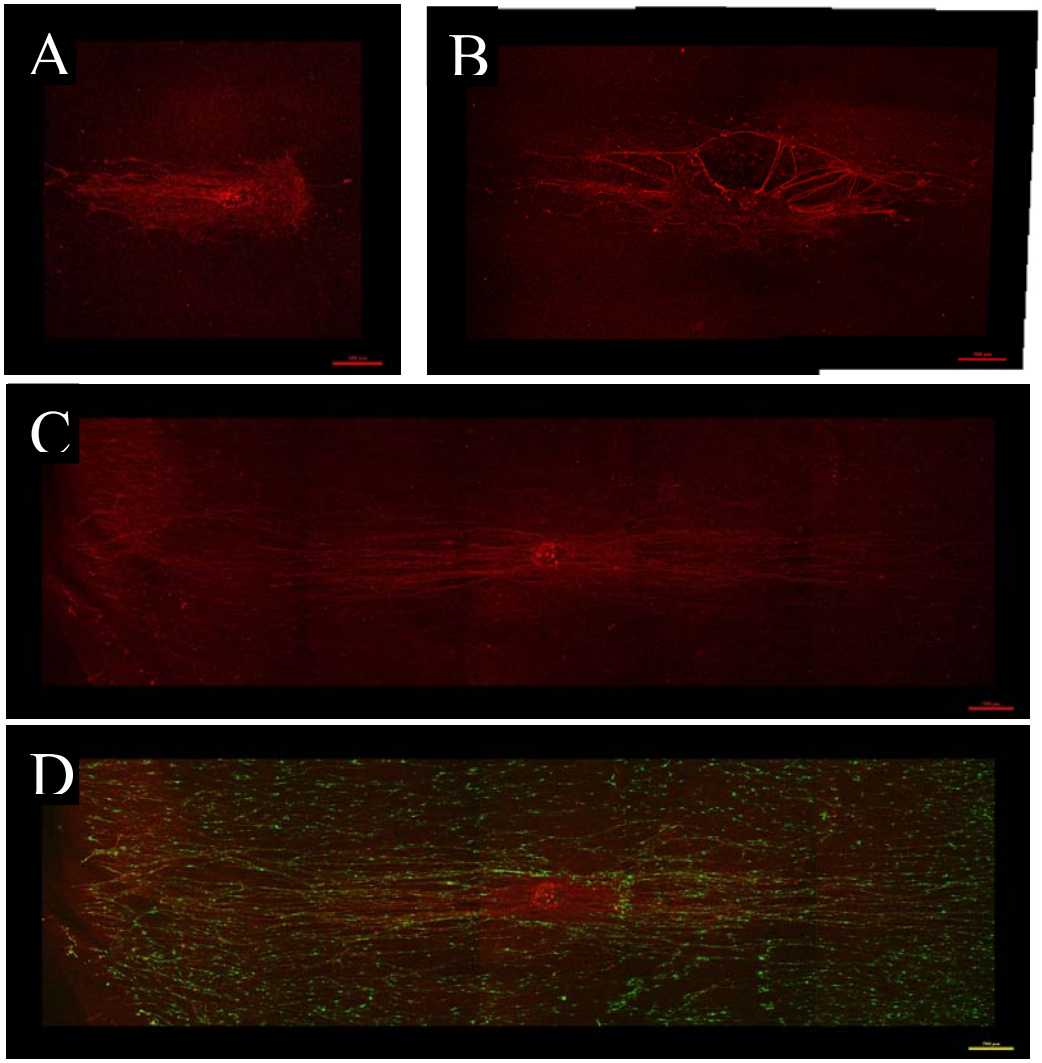

Figure 12. Purified DRG explants (Red) cultured in media with A) NGF, 0 SCs; B) NGF, BDNF and NT3, 0 SCs; C&D) representative image for groups with SCs, with or without BDNF & NT3 at 1 week. SCs are green. Figure C shows red staining only (DRG only). Figure D is a merged image showing both green and red staining (DRG with SCs). (4X, scale bar = 500 μm)

The hydrogel formulation for neurotrophin release caudal to the transplantation site was examined *in vitro*. Pluronics F127 is a FDA approved tri-block copolymer consisting of polyethylene oxide (PEO) and polypropylene oxide (PPO). It has thermoreversible gelation properties when used at concentrations of 15-20% or higher[3]. It can exist in liquid form at room temperature and rapidly transforms into hydrogel at body temperature. Because of its gelation property and biocompatibility, it has been widely used in drug delivery applications. When used as drug delivery carrier, it improves the retention of drugs locally and preserves the biological function of drugs/proteins[4]. Studies have shown that Pluronics F127 gels are compatible with neural tissues in the central nerve system when used at the concentration of 15%-20% (w/w) and prolonged drug release has been achieved with increasing concentrations of pluronics[3-7]. We evaluated the stability of Pluronics *in vitro*. 25% w/v pluronic solution was

made in DI water at 4°C and was incubated at 37ºC for the solution to gel. When immersed in PBS, the pluronics gel became soluble. For evaluating neurotrophin release, *in vitro* studies are not possible because of its rapid solubility in PBS. We will evaluate neurotrophin release using Pluronics *in vivo* in the next quarter in a pilot study.

3. Strappe, P.M., et al., *Delivery of a lentiviral vector in a Pluronic F127 gel to cells of the central nervous system.* European Journal of Pharmaceutics and Biopharmaceutics, 2005. **61**(3): p. 126-133.
4. Akash, M.S.H., et al., *Sustained Delivery of IL-1Ra from Pluronic F127-Based Thermosensitive Gel Prolongs its Therapeutic Potentials.* Pharmaceutical Research, 2012. **29**(12): p. 3475-3485.
5. Lagarce, F., et al., *Baclofen-loaded microspheres in gel suspensions for intrathecal drug delivery: In vitro and in vivo evaluation.* European Journal of Pharmaceutics and Biopharmaceutics, 2005. **61**(3): p. 171-180.
6. Sellers, D.L., et al., *Poly(lactic-co-glycolic) acid microspheres encapsulated in Pluronic F-127 prolong hirudin delivery and improve functional recovery from a demyelination lesion.* Biomaterials, 2014. **35**(31): p. 8895-8902.
7. Wu, H.-F., et al., *The promotion of functional recovery and nerve regeneration after spinal cord injury by lentiviral vectors encoding Lingo-1 shRNA delivered by Pluronic F-127.* Biomaterials, 2013. **34**(6): p. 1686-1700.

***In vivo* studies using the complete transection model in the rat.**

The complete transection model studies were performed in Dr. Bunge's laboratory at the University of Miami.

Study 1: Evaluating the influence of Matrigel in fiber filled conduits (continued from previous reporting year)

A six-week study was performed to investigate (1) improvement in SC survival by coating the fiber filled conduits (filled conduit) with Matrigel prior to transplantation; (2) improvement in SC survival and spacing between fibrous layers by injecting Matrigel with SCs at the time of transplantation to act as spacers, and (3) directional axon growth in the filled conduits. The experimental groups were as followed:

- Group **MM**: Matrigel coated and pre-loaded with GFP SCs filled conduit + injection of GFP SCs with Matrigel/DMEM/F12 between the layers at the time of transplantation.
- Group **MD**: Matrigel coated and pre-loaded with GFP SCs filled conduit + injection of GFP SCs with DMEM/F12 only between the layers at the time of transplantation.
- Group **DM**: Pre-loaded with GFP SCs filled conduit + injection of GFP SCs with Matrigel/DMEM/F12 between the layers at the time of transplantation.
- Group **DD**: Pre-loaded with GFP SCs filled conduit + injection of GFP SCs with DMEM/F12 only between the layers at the time of transplantation.

Results (additional to previous reporting year):

Myelinated and unmyelinated axons were observed in all groups (Figure 13.C) but the numbers of myelinated and unmyelinated axons were not significantly different (Figure 13.A). Group DD had the lowest mean of myelinated axons suggesting that the presence of Matrigel, either pre-coated or injected between the layers, promoted axon regeneration/myelination (Figure 13.A). Number of blood

vessels among the groups were similar suggesting Matrigel did not influence blood vessel formation (Figure 13.B).

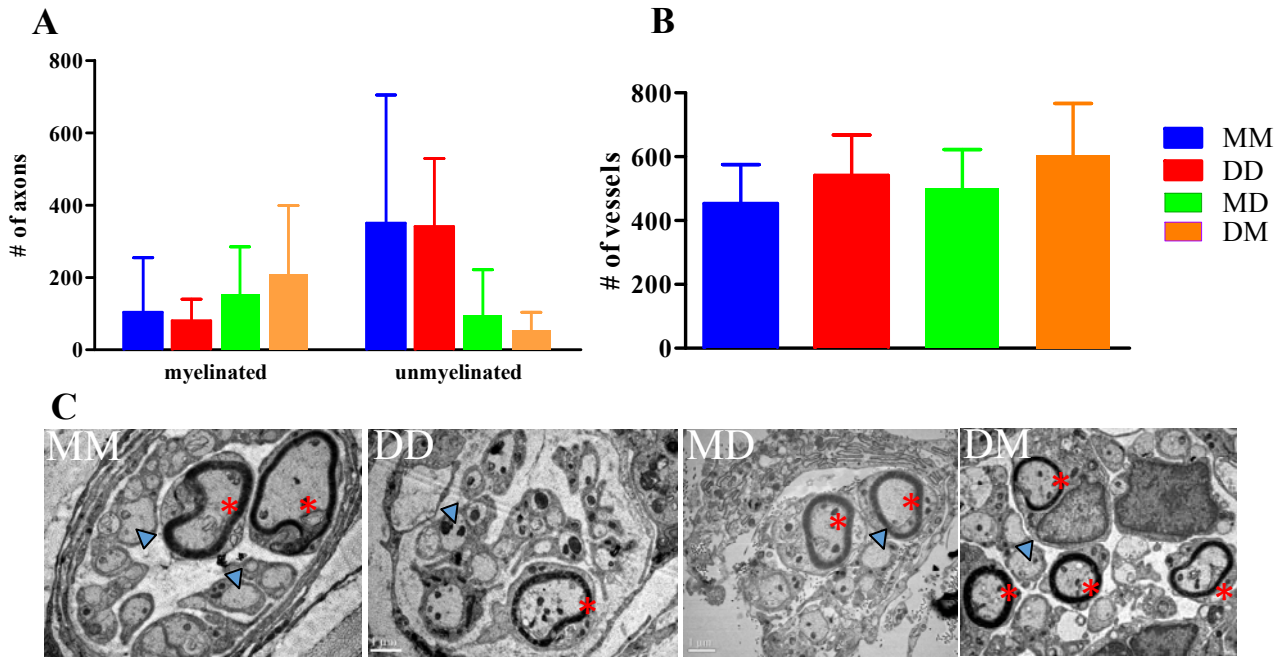

Figure 13. Quantitation of myelinated and unmyelinated axons (A) and blood vessels (B) for groups MM, DD, MD, and DM (mean±std). Transmission electron microscopy (TEM) images showing myelinated (*) and unmyelinated (blue triangle) axons in groups MM, DD, MD, and DM (C).

There were no qualitative differences in the initial assessment of green fluorescent protein (GFP)-SCs and regenerated axons in sagittal section. After removing 1 mm in the center of the bridge region for plastic sections, the remaining bridge tissue was cut into 20 um cross-sections and stained for GFP and neurofilament (NF, axons). GFP^+ and NF^+ fluorescent areas in the cross-section were directly associated with the amount of GFP-SCs and regenerated axons in the bridge. GFP-SCs attached to the interior wall of the conduits and along the layers in all groups (Figure 14.C). Regenerated axons were associated with GFP-SCs and were along the layers in the conduit (Figure 14.C). No significant differences were observed in GFP^+ areas (Figure 14.A). However, the means of group MM and DM were higher compared to group DD and MD suggesting that injecting Matrigel between the layers might enhance SC survival. NF^+ areas were similar among all groups regardless of the presence of Matrigel.

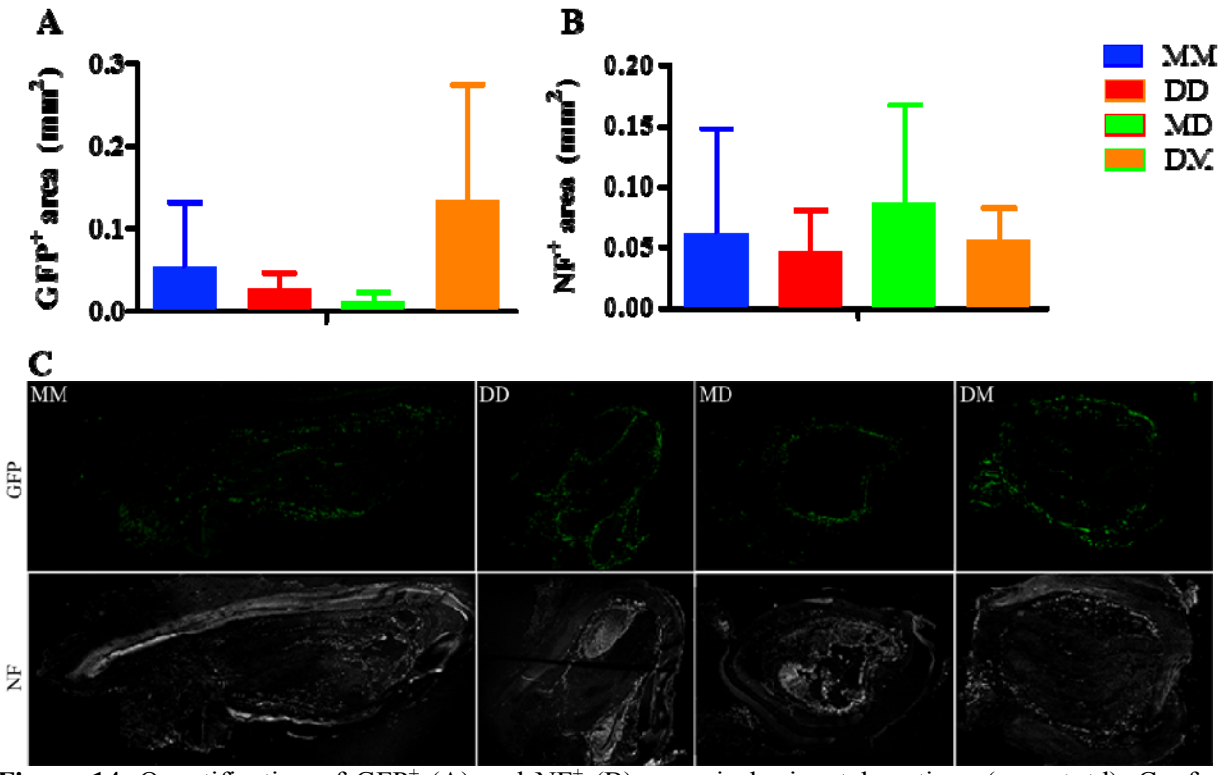

Figure 14. Quantification of GFP^+ (A) and NF^+ (B) areas in horizontal sections (mean±std). Confocal fluorescent images of 20µm cross sections stained with GFP (transplanted SCs, green) and NF (axons, white) antibodies of groups MM, DD, MD, and DM six weeks post-transplantation(C).

Study 2: Evaluating the influence of Matrigel in hollow vs filled conduits

A six-week study was performed to investigate the influence of Matrigel in hollow vs fiber filled conduits on SC survival and myelination and axon regeneration. The fibrous layers were optimized for fiber diameter and interfiber spacing, as reported in year 1. The experimental groups of the 6-week study were:

- Hollow: Hollow conduit + injection of GFP-SCs with Matrigel/DMEM/F12 at the time of transplantation.
- Filled: Filled conduit + injection of GFP-SCs with Matrigel/DMEM/F12 between the fibrous layers at the time of transplantation.

The outcome measures were:

- Behavior: weekly BBB test.
- Immunostaining for: GFAP (glial fibrillary acidic protein, astrocyte), GFP (transplanted SCs), P75 (NGF receptor, endogenous SCs), Iba1 (calcium-binding protein, macrophage/microglia), PDGFrB (platelet-derived growth factor receptor beta, fibroblast), NF (neurofilament, regenerated axon), DβH (dopamine beta hydroxylase, dopaminergic axons), 5HT (serotonergic axons), CGRP (calcitonin gene-related peptide, sensory axon), and Reca1 (blood vessels). Quantify the fluorescent positive area of each staining.
- Plastic: Quantify the number of vessels, myelinated axons and unmyelinated axons.

Results

Frozen horizontal sections were stained with GFP, GFAP, RT97, and PDGFrB antibodies. Similar GFP-SC survival was observed in hollow (Figure 15.A) and filled (Figure 15.B) conduits but GFP-SCs were more evenly distributed along the length of the latter conduit. Directed $RT97^{+}$ axon growth was observed along the layers throughout the entire length of the filled conduits (Figure 15.B and insert, within the yellow dashed lines between the layers). The layers were designed to have larger interfiber spacing (as previously reported) than in previous studies to allow for better cell and axon infiltration within the conduit. GFP-SCs and axons were observed within the layers (Figure 15.B insert) as compared to previous study 1. Sporadic $RT97^{+}$ axonal growth without direction was observed in the hollow conduits (Figure 15.A and insert).

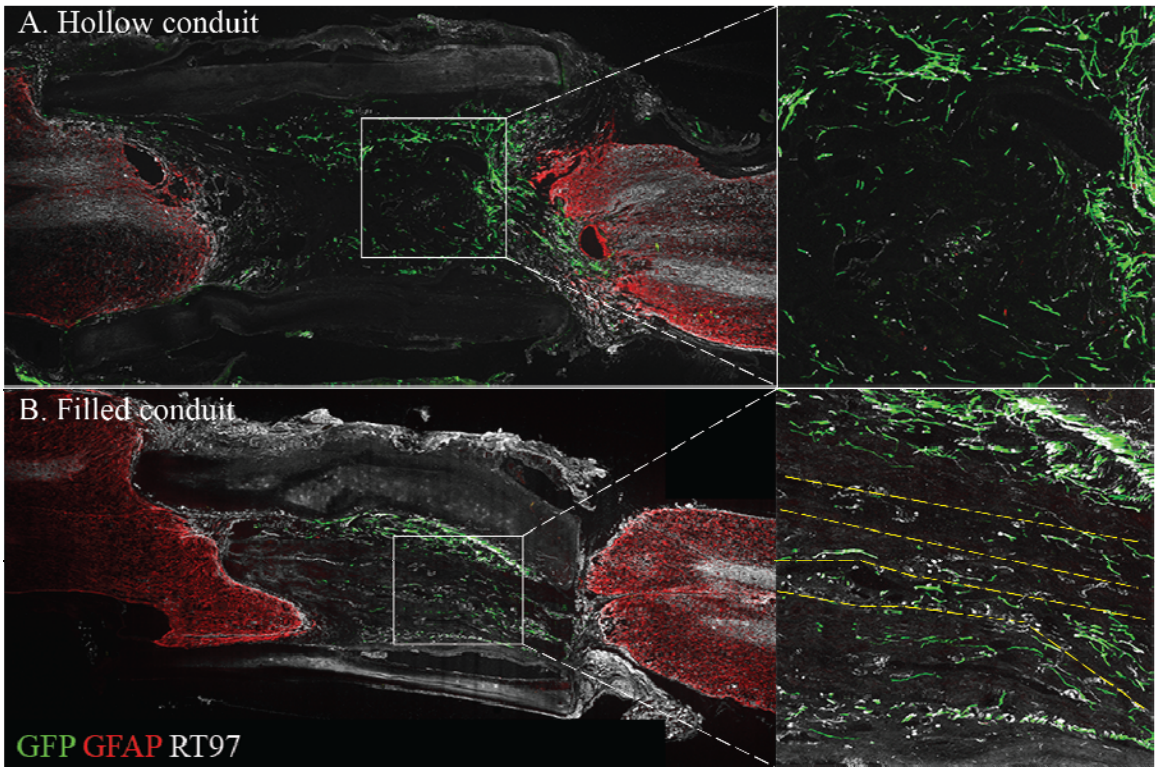

Figure 15. Confocal fluorescent images of 20 μm horizontal sections stained with GFP, GFAP, and RT97 antibodies of hollow (A) or filled (B) conduits six weeks post transplantation. Higher magnification inserts show the areas of interest.

A control (transection only) was performed along with the experimental groups to investigate the presence of fibroblast after a complete transection. Fibroblasts ($PDGFr\beta^{+}$) were found in the grey matter of the caudal stomp and robustly at the injury site of a transected spinal cord without treatment (Figure 16.A). They are also found throughout the hollow (Figure 16.B) and filled (Figure 16.C) conduits. Fibroblasts have a similar presence in the caudal stomp in the hollow conduits as the untreated group. The significance of fibroblast presence in the caudal stomp will be further investigated.

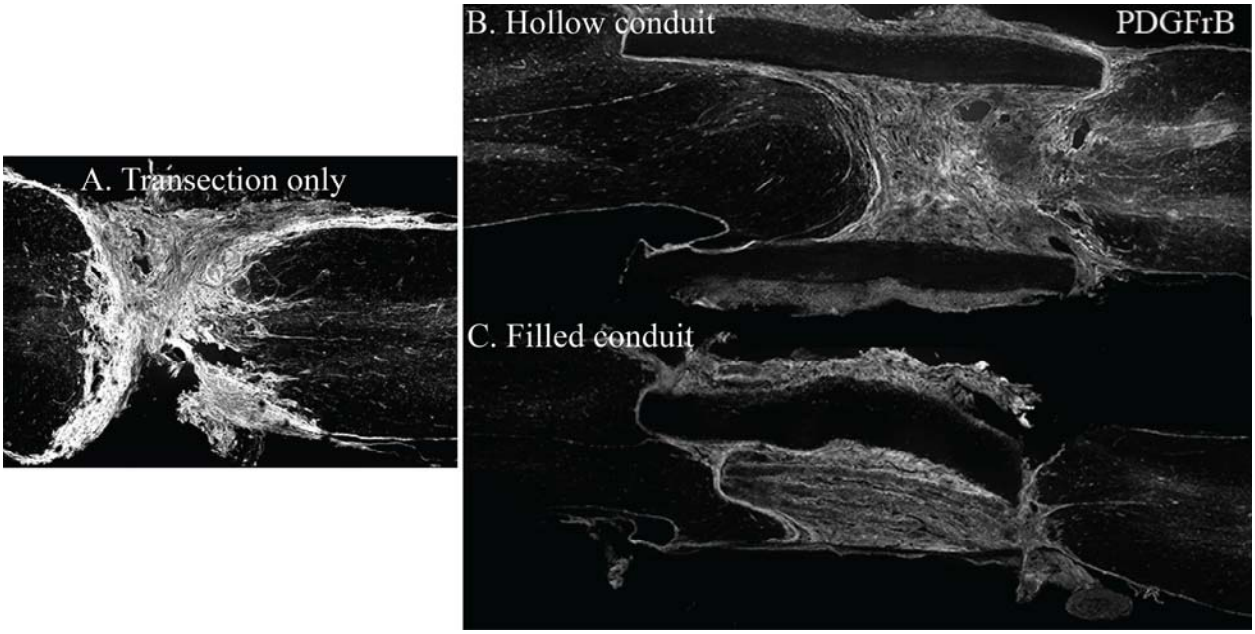

Figure 16. Confocal fluorescent images of 20μm horizontal sections stained with PDGFrβ (fibroblast) antibody of transection only (A), hollow (B) or filled (C) conduits six weeks post transplantation.

Conduit structure was maintained in fiber filled (Figure 17.B) conduits better than the hollow (Figure 17.A) conduits six weeks post transplantation. Matrigel degradation in the hollow conduits may have been contributed to the conduit structural deformation. Both conduits showed abundant vessel formation and axon myelination (Figure 17.C and D). There were no significant differences in the number of myelinated and unmyelinated axons (Figure 17.E and F). The means of myelinated and unmyelinated axons were slightly higher in hollow conduits suggested that there may be more axons regenerated into the bridge. Number of axons at the caudal end of the bridge will be further investigated to determine if filled conduits guided more axons into the caudal end as compared to hollow conduits. Based on studies from last year, we expect similar findings. There were no significant differences in the number of blood vessels between groups (Figure 17.G). The number of blood vessels in filled conduit in this study was similar to the previous study (group DM, Figure 13.B). Weekly BBB scores (Figure 18) did not show significant differences in hindlimb movement recovery between animals receiving hollow or filled conduits with SCs/Matrigel. In summary, the optimized fibrous layers, having larger interfiber spacing than in previous studies, allow for better infiltration of axons and SCs throughout the structure than the previous design and provide guidance for axonal growth along the length of the layers/fibers in the conduit. This design was used in the following pilot study for neurotrophin release.

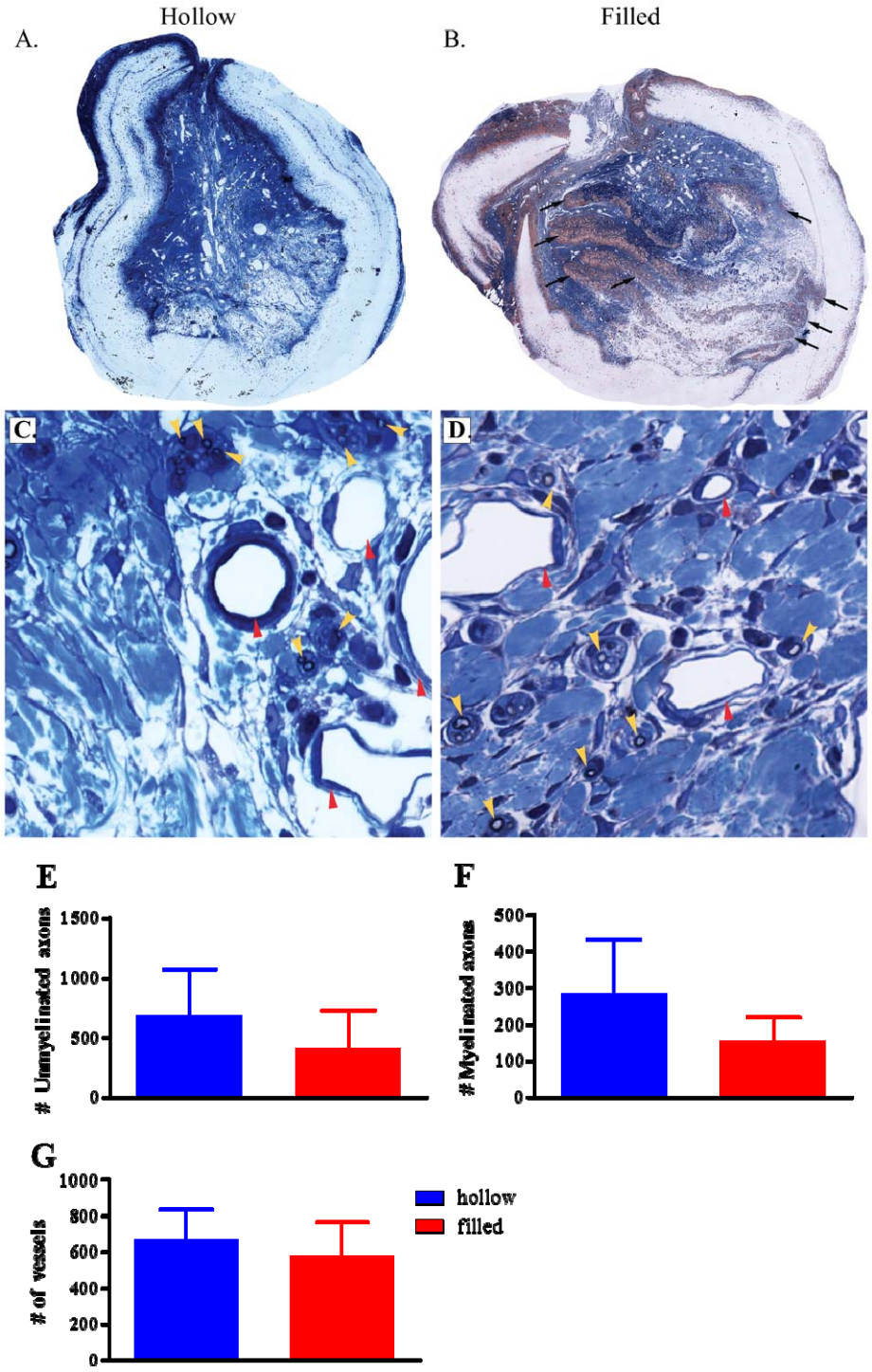

Figure 17. Bright field images of 10um transverse plastic sections stained with toluidine blue of hollow (A) and filled (B) conduits six weeks post transplantation. Layers are indicated by black arrows. Areas of interest showing vessel formation (red arrow head) and myelinated axons (yellow arrow head) in hollow (C) and filled (D) conduits. Quantitation of unmyelinated (E) and myelinated (F) axons and blood vessels (G) for hollow vs filled conduits.

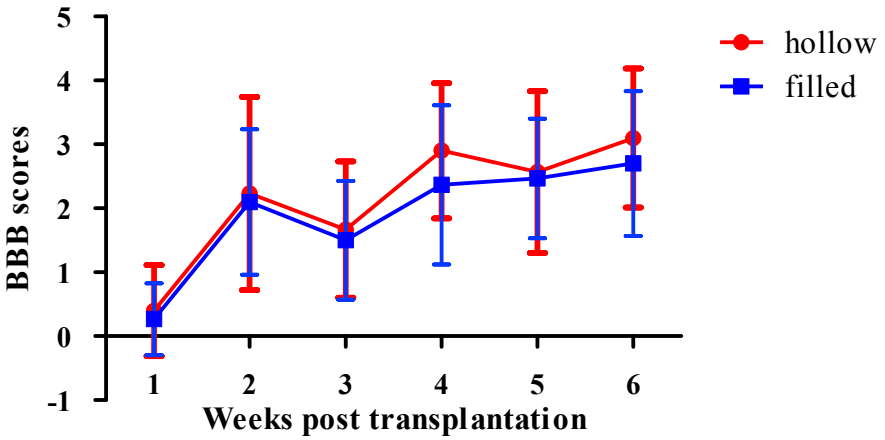

Figure 18. Weekly BBB scores post transplantation of hollow and filled conduits.

Study 3: Filled conduits with BDNF/NT3 release from the fibrous layers

A six-week study was performed to investigate improvement of SC survival and axon regeneration using neurotrophin releasing piezoelectric conduits with SCs. We hypothesize the controlled release of BDNF and NT3 from the fibrous layers in the conduits will promote axon regeneration. Matrigel has well known properties in promoting cell survival and axon regeneration. However, in our previous studies, Matrigel containing conduits did not have a significant effect on axon regeneration in comparison to conduits without Matrigel. Therefore, we were evaluating conduits with or without Matrigel and in combination with BDNF and NT3 release. The outcome of this pilot study will determine whether Matrigel will be used in the full study proposed for year 3. The experimental groups were:

- +BDNF/NT3/+Matrigel: Filled conduits with BDNF/NT3 release + injection of GFP-SC/DMEM/F12/Matrigel
- -BDNF/NT3/-Matrigel: Filled conduits without BDNF/NT3 release + injection of GFP-SC/DMEM/F12/Matrigel
- +BDNF/NT3/-Matrigel: Filled conduits with BDNF/NT3 release + injection of GFP-SC/DMEM/F12
- -BDNF/NT3/-Matrigel: Filled conduits without BDNF/NT3 release + injection of GFP-SC/DMEM/F12

The outcome measures were:

- Behavior: weekly BBB and inclined plane tests.
- Immunostaining for GFAP, GFP, P75, Iba1, PDGFrB, NF, DβH, 5HT, CGRP, and Reca1. Quantify the fluorescent positive area of each stain.
- Plastic: Quantity the number of vessels, myelinated axons and unmyelinated axons.

Results

There were no significant differences in weekly BBB scores among the groups, although by 6 weeks post transplantation, the BDNF/NT3 releasing conduit with Matrigel group had the highest score (Figure 19). The inclined plane test was performed by placing the animal on an inclined plane facing four directions: up, down, left, and right, and the highest angle that it could stay without falling in each direction for five seconds was recorded. The average inclined plane angle of four directions was

calculated and its percentage recovery compared to the baseline was calculated weekly. The average percentage recovery in animals receiving BDNF/NT3 releasing conduits with Matrigel was significantly higher than in animals receiving BDNF/NT3 releasing conduits without Matrigel at six weeks post transplantation (Figure 20).

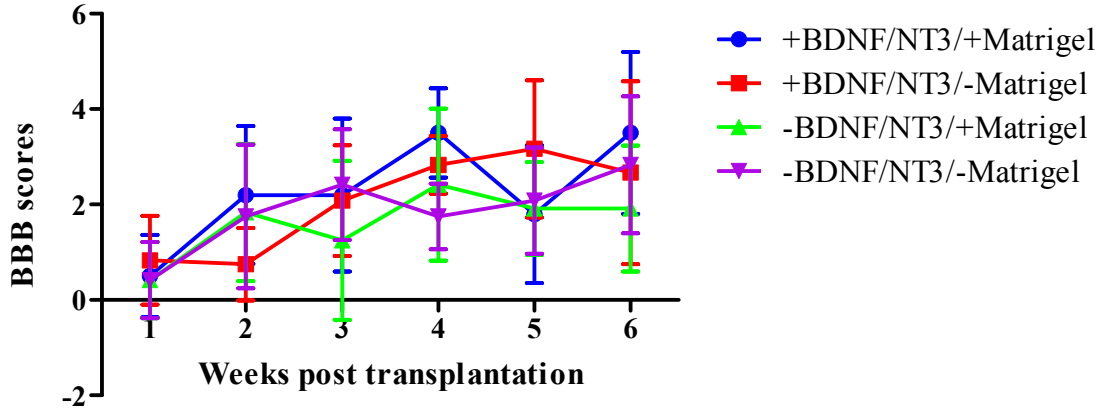

Figure 19. Weekly BBB scores post transplantation of animals receiving filled conduits with or without BDNF/NT3 controlled release and with or without Matrigel.

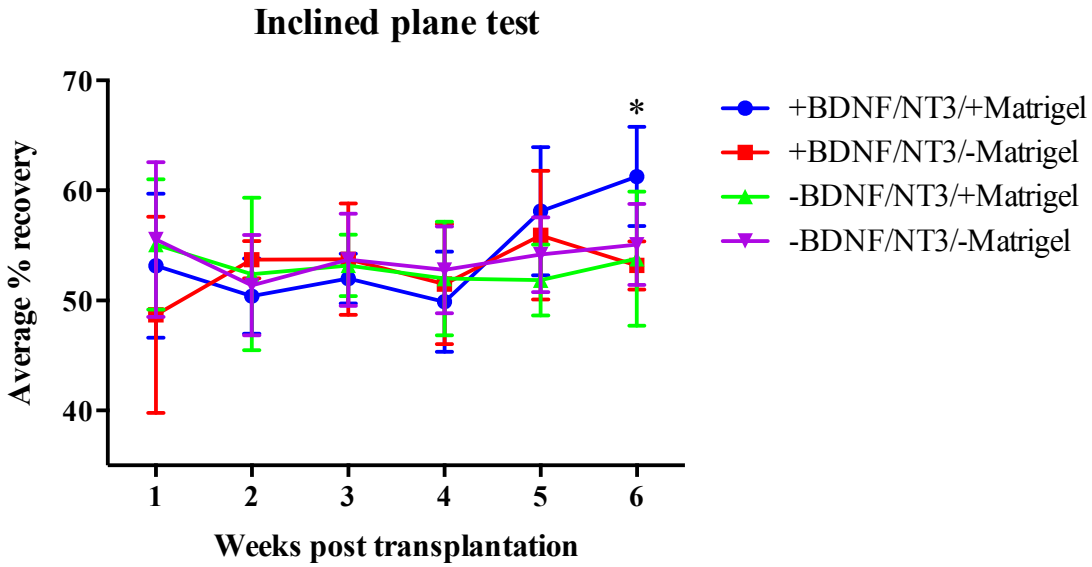

Figure 20. Average weekly percentage of recovery in inclined plane test compared to baseline. *At six weeks post transplantation, animals receiving BDNF/NT3 releasing conduits with Matrigel had recovered significantly better compared to animals receiving BDNF/NT3 releasing conduits without Matrigel (p=0.05, ANOVA with post-hoc Tukey's test).

Similar GFP-SCs survival was observed in all conduit groups (Figure 21.C), with no significant differences detected among the groups (Figure 21.A). Regenerated axons were observed in all conduit groups (Figure 21.C), with no significant differences detected among the group (Figure 21.B). However, groups with Matrigel had a higher mean for NF^+ area where the highest mean was for BDNF/NT3 releasing conduit with Matrigel. The lowest mean for NF^+ area was observed in the group without Matrigel and without BDNF/NT3 release.

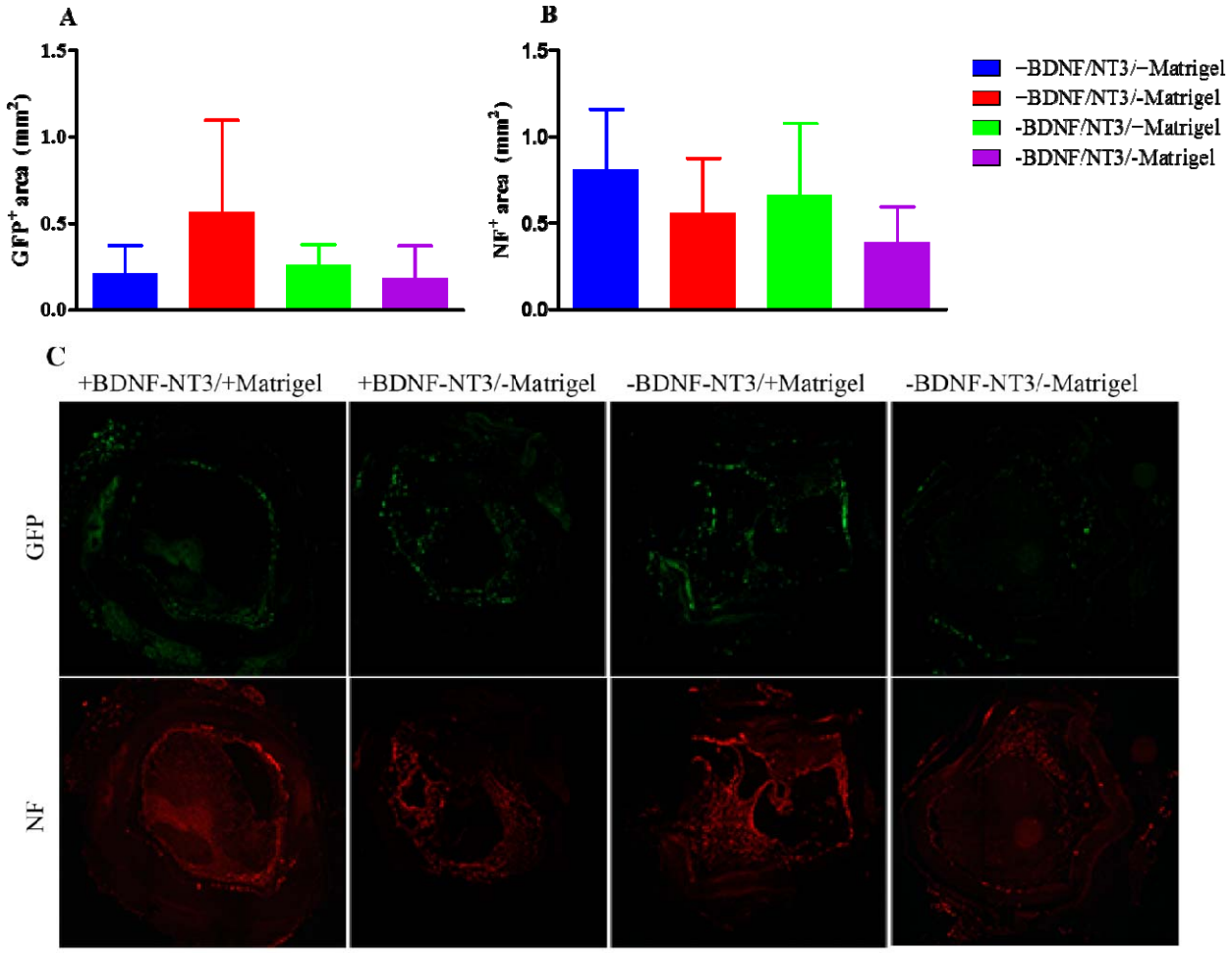

Figure 21. Quantification of GFP^+ (A) and NF^+ (B) fluorescent area in horizontal sections (mean±std) of groups +BDNF/NT3/+Matrigel, +BDNF/NT3/-Matrigel, -BDNF/NT3/+Matrigel, and -BDNF/NT3/-Matrigel. Confocal fluorescent images of 25 µm cross sections stained with GFP and NF antibodies of groups +BDNF/NT3/+Matrigel, +BDNF/NT3/-Matrigel, -BDNF/NT3/+Matrigel, and -BDNF/NT3/-Matrigel six weeks post-transplantation (C).

The means of GFP^+ area in filled conduits without BDNF/NT3 release and with or without Matrigel were higher than the means of GFP^+ area in study 1 (Figure 14.A, group DM and DD) demonstrating that new design of the fibrous layers improved SC engraftment. In this study, the layers had more interfiber spacing as compared to study 1. The layers were also thinner in this study. Although no significant differences were observed in NF^+ area in this study, they were almost 10-fold higher than in study 1 for all groups (Figure 14.B), with BDNF/NT3 release with Matrigel having the highest area. The increase in NF+ area can be attributed to the presence of BDNF/NT3 release in combination with Matrigel and also the new design of the fibrous layers especially for conduits without BDNF/NT3 release. Based on findings thus far, we will continue to use Matrigel in our conduits.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
For next quarter, we will conduct the pilot *in vivo* study examining the use of the neurotrophin release caudal to the transplant prior to performing the large *in vivo* study in order to characterize neurotrophin release from the Pluronics gel and axonal regeneration throughout the conduit and caudal to the transplantation site. We also will finish analyzing the data in the *in vivo* studies 1 and 2 and prepare a manuscript. For the *in vitro* studies, the cell culture work using neurotrophin releasing scaffolds in co-culture with DRGs and SCs will be completed. A manuscript also is in preparation for the *in vitro* studies from years 1 and 2 evaluating neurite extension with SCs in co-culture on PVDF-TrFE scaffolds.

4. IMPACT
What was the impact on the development of the principal disciplines of the project?
Nothing to Report.

What was the impact on other discipline?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGE/PROBLEMS
Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS
Publications, conference papers, and presentations

Journal publications.
Peer-reviewed journal publication:
Lee, Y.S., Wu, S., Arinzeh, T., Bunge, B. (2016), Enhanced noradrenergic axon regeneration into Schwann cell-filled PVDF-TrFE conduits after complete spinal cord transection. *Biotechnol Bioeng*, DOI:10.1002/bit.26088. (epub ahead of print)

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Conference paper:
Lee, Y.-S., Wu, S., Livingston Arinzeh, T.*, Bunge, M.B. (2016), Schwann cell loaded PVDF-TrFE scaffolds promote axon regeneration after spinal cord repair. Biomedical Engineering Society Annual conference, Minneapolis, MN. *Podium Presentation

Lee, Y.-S.*, Wu, S., Livingston Arinzeh, T., Bunge, M.B. (2016), Schwann cell-laden PVDF-TrFE Conduits for Spinal Cord Repair. World Congress of Biomaterials. DOI: 10.3389/conf.FBIOE.2016.01.01741. *Podium Presentation

Wu, S.*, Lee, Y.-S., Bunge, M.B., Arinzeh, T. (2016), Investigating Schwann cell growth and neurite extension on PVDF-TrFE scaffolds *in vitro*. World Congress of Biomaterials. DOI: 10.3389/conf.FBIOE.2016.01.01273. *Poster Presentation

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and /or licenses
Nothing to Report.

Other products
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Name: Treena Arinzeh
Project Role: PI
Nearest person month worked: 4
Contribution to Project: Dr. Arinzeh oversees all aspects of the experiments in the project, determines study designs, works with collaborators, interprets data and presents and publishes findings.

Name: Mary Bunge
Project Role: Qualified Collaborator/Co-I
Nearest person month worked: 4

Contribution to Project: She oversees all *in vivo* experiments using the complete transection model and collaborates on the in vitro studies. She determines study designs in collaboration with the PI, interprets data and contributes to the publishing and presenting of the findings.

Name: Mesut Sahin
Project Role: Collaborator/Co-I
Nearest person month worked: 0.5
Contribution to Project: He oversees the *in vivo* experiments measuring the electrical activity of the piezoelectric materials. He determines the recording device/setup and interprets the data in collaboration with the PI.

Name: Yee-Shuan Lee
Project Role: Postdoctoral Fellow
Nearest person month worked: 12
Contribution to Project: She performs all in vivo experiments using the complete transection model and analyses.

Name: Siliang Wu
Project Role: PhD student
Nearest person month worked: 12
Contribution to Project: He performs all in vitro experiments, including materials characterization and SC cell viability and growth studies.

Name: Sinan Gok
Project Role: PhD student
Nearest person month worked: 3
Contribution to Project: He performs the piezoelectric characterization of the piezoelectric material in vivo.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Collaborative awards: N.A.
Quad Charts: N.A.

9. APPENDICES

Lee, Y.S., Wu, S., Arinzeh, T., Bunge, B. (2016), Enhanced noradrenergic axon regeneration into Schwann cell-filled PVDF-TrFE conduits after complete spinal cord transection. *Biotechnol Bioeng*, DOI:10.1002/bit.26088. (epub ahead of print)

Lee, Y.-S., Wu, S., Livingston Arinzeh, T.*, Bunge, M.B. (2016), Schwann cell loaded PVDF-TrFE scaffolds promote axon regeneration after spinal cord repair. Biomedical Engineering Society Annual conference, Minneapolis, MN. *Podium Presentation

Lee, Y.-S.*, Wu, S., Livingston Arinzeh, T., Bunge, M.B. (2016), Schwann cell-laden PVDF-TrFE Conduits for Spinal Cord Repair. World Congress of Biomaterials. DOI: 10.3389/conf.FBIOE.2016.01.01741. *Podium Presentation

Wu, S.*, Lee, Y.-S., Bunge, M.B., Arinzeh, T. (2016), Investigating Schwann cell growth and neurite extension on PVDF-TrFE scaffolds *in vitro*. World Congress of Biomaterials. DOI: 10.3389/conf.FBIOE.2016.01.01273. *Poster Presentation

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Enhanced Noradrenergic Axon Regeneration Into Schwann Cell-Filled PVDF-TrFE Conduits After Complete Spinal Cord Transection

Yee-Shuan Lee,[1] Siliang Wu,[2] Treena Livingston Arinzeh,[3] Mary Bartlett Bunge[1,4,5]

[1]The Miami Project to Cure Paralysis, Lois Pope LIFE Center, University of Miami Miller School of Medicine, P.O. Box 016960, Mail locator R-48, Miami, Florida 33101; telephone: 305-243-4596; fax: 305-243-3923; e-mail: mbunge@miami.edu

[2]Department of Material Science and Engineering, New Jersey Institute of Technology, Newark, New Jersey

[3]Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, New Jersey

[4]Department of Cell Biology, University of Miami Miller School of Medicine, Miami, Florida 33101

[5]Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, Florida 33101

ABSTRACT: Schwann cell (SC) transplantation has been utilized for spinal cord repair and demonstrated to be a promising therapeutic strategy. In this study, we investigated the feasibility of combining SC transplantation with novel conduits to bridge the completely transected adult rat spinal cord. This is the first and initial study to evaluate the potential of using a fibrous piezoelectric polyvinylidene fluoride trifluoroethylene (PVDF-TrFE) conduit with SCs for spinal cord repair. PVDF-TrFE has been shown to enhance neurite growth in vitro and peripheral nerve repair in vivo. In this study, SCs adhered and proliferated when seeded onto PVDF-TrFE scaffolds in vitro. SCs and PVDF-TrFE conduits, consisting of random or aligned fibrous inner walls, were transplanted into transected rat spinal cords for 3 weeks to examine early repair. Glial fibrillary acidic protein $(GFAP)^+$ astrocyte processes and GFP (green fluorescent protein)-SCs were interdigitated at both rostral and caudal spinal cord/SC transplant interfaces in both types of conduits, indicative of permissivity to axon growth. More noradrenergic/$D\beta H^+$ (dopamine-beta-hydroxylase) brainstem axons regenerated across the transplant when greater numbers of $GFAP^+$ astrocyte processes were present. Aligned conduits promoted extension of $D\beta H^+$ axons and $GFAP^+$ processes farther into the transplant than random conduits. Sensory $CGRP^+$ (calcitonin gene-related peptide) axons were present at the caudal interface. Blood vessels formed throughout the transplant in both conduits. This study demonstrates that PVDF-TrFE conduits harboring SCs are promising for spinal cord repair and deserve further investigation.

Biotechnol. Bioeng. 2016;9999: 1–13.

KEYWORDS: Schwann cells; spinal cord injury; electrospinning; conduit; PVDF-TrFE; aligned fibers

Introduction

Traumatic spinal cord injury (SCI) initiates a cascade of biochemical and cellular events at the site of injury to create a hostile environment for repair and regeneration (Fawcett and Asher, 1999; Schwab and Bartholdi, 1996; Silver and Miller, 2004). Large cysts developing over time in contused spinal cord contribute to a milieu that is unfavorable for the regrowth of axons needed to re-establish communication across the injury. In interrupted spinal cord, interventions are needed to provide bridges across the injury consisting of a suitable milieu that promotes repair and axon regeneration (Fortun et al., 2009). Combining cells with scaffolds and additional signals is a promising strategy for providing an improved environment for repair.

Schwann cells (SCs) play an essential role for axon regeneration in peripheral nerve. They secrete numerous neurotrophic factors and extracellular matrix proteins that stimulate axon extension

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Contract grant sponsor: NJCSCR
Contract grant number: CSCR14FEL004
Contract grant sponsor: Miami Project to Cure Paralysis
Contract grant sponsor: Buoniconti Fund
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(Nomura et al., 2006). SCs transplanted into the contused rat spinal cord reduce cyst size, provide neuroprotection for tissue around the injury, promote propriospinal and supraspinal axon regeneration, myelinate those axons, and may improve functional recovery (Fortun et al., 2009; Tetzlaff et al., 2011). Repair is enhanced when SCs are combined with additional treatments (Fortun et al., 2009; Kanno et al., 2015; Tetzlaff et al., 2011). Of the varied cell types transplanted to test their efficacy in SCI animal models, SCs have been among the most successful.

Due to the accessibility of human SCs in a peripheral nerve biopsy and the development of tissue culture techniques to isolate, purify and increase the number of SCs from biopsies, clinical trials have been possible. A strong advantage of the use of human SCs is that they can be autologously transplanted. SC transplantation clinical trials have been initiated in Iran (Oraee-Yazdani et al., 2016; Saberi et al., 2008, 2011; Yazdani et al., 2013), China (Chen et al., 2014; Zhou et al., 2012), and the United States [Guest et al., 2013; (www.clinicaltrials.gov NCT01739023)]. The SC trials have been shown to be safe and free of adverse events and to lead to some improvements after SCI. In the United States, there is a new SC transplantation clinical trial for subjects with chronic SCI (www.clinicaltrials.gov NCT02354625).

In peripheral nerve, a gap of only a few mm can be repaired without intervention due to the migration of SCs with accompanying regenerating axons into the gap. In contrast, because gaps in the injured spinal cord are not repaired without intervention, the results are devastating. Transplants to bridge the human injury site are needed. In our previous completely transected rat spinal cord studies, SC transplants were positioned between the cord stumps, held in place by a polyacrylonitrile/polyvinylchloride (PAN/PVC) conduit (Xu et al., 1997). Because the PAN/PVC conduits are bioinert, they are not considered to enhance axon regeneration or SC survival. A variety of scaffolds are being tested (summarized by Straley et al., 2010), some in combination with cells (summarized by Han and Cheung, 2011 and Kubinova and Sykova, 2012), for their ability to promote spinal cord repair. A multifunctioning conduit that can both enhance survival of transplanted cells and promote axon regeneration is needed for an effective therapy for the injured spinal cord.

Conduits made of polyvinylidene fluoride trifluoroethylene (PVDF-TrFE) may hold promise because PVDF-TrFE has well-known piezoelectric characteristics and is biocompatible (Rajabi et al., 2015). PVDF and its co-polymers are currently used in clinical products or products in clinical trials (XIENCE V® Everolimus Eluting Coronary Stent System; www.clinicaltrials.gov NCT02407145). Piezoelectric materials produce electrical activity in response to minute deformations. Body movement and change in posture result in spinal cord stresses and strains (Harrison et al., 1999a,b,c), which may cause deformation in the conduit activating the piezoelectric property. PVDF-TrFE has been shown to promote neuronal differentiation of mouse neuroblastoma cells (Valentini et al., 1992) and human neural stem cells (Lee and Arinzeh, 2012) and neurite outgrowth of rat dorsal root ganglia (Lee et al., 2011) in vitro. In sciatic nerve repair, the use of PVDF-TrFE conduits increased the number of myelinated regenerated axons more than a sciatic nerve graft (Aebischer et al., 1987a; Fine et al., 1991).

Conduits used in our study were fabricated by electrospinning, a common and efficient method to prepare fibrous scaffolds for nerve repair (Biazar and Keshel, 2013; Hurtado et al., 2011) and to promote axon growth due to contact guidance (He et al., 2010; Lee et al., 2009, 2011; Liu et al., 2010; Qu et al., 2013; Weightman et al., 2014). Electrospun PVDF-TrFE scaffolds have previously been shown to maintain their piezoelectric crystal structure after electrospinning and the crystallinity can be further enhanced by annealing (Lee et al., 2011). In addition, recent studies have demonstrated the potential of electrospun PVDF-TrFE as a high performance piezoelectric device (Baniasadi et al., 2016; Persano et al., 2013).

The study reported here is the first and initial study to evaluate the combination of SCs with fibrous PVDF-TrFE conduits to repair a completely transected rat spinal cord. The goal of our study was to evaluate the feasibility of using PVDF-TrFE aligned or random fibrous conduits to transplant SCs and promote axon regeneration. The advantage of aligned conduits is to enable contact guidance along the rostral-caudal axis of the conduit to promote neurite extension. This study demonstrates SC viability in vitro and survival of transplanted SCs and regeneration of axons within the conduits in vivo. Axons regenerated more robustly and SCs were more uniformly distributed in SC transplants in aligned than random conduits.

Materials and Methods

Scaffold Fabrication

Electrospinning and Conduit Assembly

Electrospinning was conducted using a setup and parameters as previously described (Greiner and Wendorff, 2007; Lee et al., 2011; Weber et al., 2010). 15% (w/v) of poly (vinylidene fluoride trifluoroethylene) (65/35) (PVDF-TrFE; Solvay Solexis Inc., Thorofare, NJ) in methyl ethyl ketone (MEK; Avantor Performance Materials, Inc., Center Valley, PA) was loaded into a syringe with a 22 gauge needle. The solution was ejected out of the syringe at 2 mL/h using a syringe pump. A high-voltage power source was used to generate a voltage of 28 kV that was applied to the tip of the needle of the syringe and the collector on which the fibers were deposited was grounded. The distance between the tip of the needle and the collector was kept at 35 cm. Random fibers were collected on a grounded metal plate. Aligned fibers were collected on a fast-rotating drum. Additional fibers were collected randomly on top of the aligned fibers to achieve the thickness required for conduit formation. Both types of scaffolds were annealed to enhance the presence of the piezoelectric crystal phase (Lee et al., 2011); the electrospun scaffolds were kept at 135°C for 96 h and quenched with ice water. All samples were kept in a chemical hood for at least 72 h to ensure that solvents had evaporated before using for cell culture studies.

For in vitro experiments, the scaffolds were cut into 6 mm disks and sterilized by ultraviolet irradiation. For in vivo experiments, the scaffolds were rolled into a hollow conduit with an inner diameter between 2.4–2.7 mm and outer diameter between 2.5–2.8 mm by sealing the edges with 15% (w/v) PVDF-TrFE solution (Fig. 1A). For the aligned fibrous conduits, the aligned fibers were longitudinally

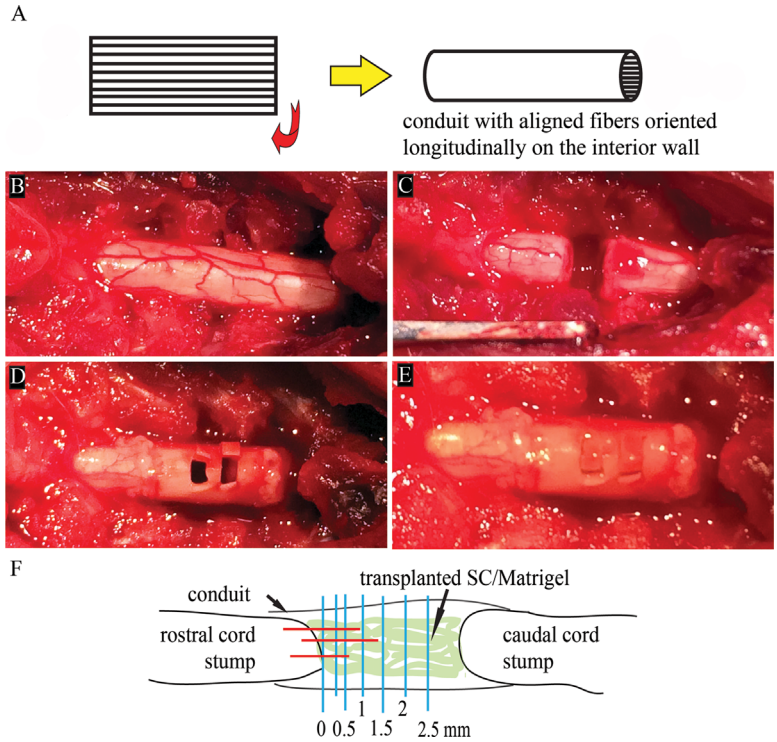

Figure 1. An aligned fibrous scaffold was rolled and its edges sealed to form a conduit. The aligned fibers were oriented longitudinally on the interior wall of the conduit (**A**). Dorsal exposure of a rat spinal cord from T7 to T9 after a multi-level laminectomy (**B**). A gap was created after transection at T8 (**C**), and a conduit inserted between the rostral and caudal stumps. Pre-cut windows on the dorsal surface allowed the injection of SCs (**D**); windows were closed after injection (**E**). Illustration of the line-transect method utilized for quantifying immunostained DβH$^+$ axons and GFAP$^+$ astrocyte processes (**F**). The rostral spinal cord/SC bridge interface was defined by the dense expression of GFAP$^+$ astrocyte somata. The 0 line, indicated at the rostral interface, was defined for each animal on the tissue section where the rostral spinal cord extended farthest into the conduit. The distance between the 0 line and the rostral end of the conduit in this section was used to define the location of the 0 line in other sections from the same animal. Dorsoventral lines (light blue) were drawn at 0.25, 0.5, 1, 1.5, 2, and 2.5 mm caudal to the 0 line on each tissue section (visualized by Hoechst staining) within the conduit. The red lines represent axons that cross the rostral interface into the SC transplant. The numbers of DβH$^+$ axons and GFAP$^+$ astrocyte processes that crossed each dorsoventral line were counted.

aligned on the interior surface and the random fibers were on the exterior surface (Fig. 1A). The conduits were placed in a chemical hood for at least 48 h to allow solvent evaporation prior to sterilization. The conduits were cut into 5 mm lengths. Two small windows were created by cutting three sides of a rectangle, as shown in Figure 1D, to create an opening for GFP-SC/Matrigel injection into the conduit as described in a later section. These conduits, sterilized with 75% ethanol followed by several rinses of phosphate buffered saline (PBS), were kept in PBS until transplantation.

Scaffold Morphology and Characterization

Fiber morphology, diameter, alignment and inter-fiber spacing were characterized using scanning electron microscopy (SEM) (LEO 1530 Gemini, Zeiss, Oberkochen, Germany). Two samples from each type of scaffold were prepared for SEM and four images were taken per sample. All measurements were performed using ImageJ software (1.47V) ($n = 80$/scaffold type) as described elsewhere (Lee et al., 2011). Fiber diameter and alignment were calculated as previously described (Lee et al., 2011). The interfiber spacing was determined by measuring the distance between two parallel/adjacent fibers or between the intersection of two fibers and the opposite fiber. The porosity was determined by the difference in density between the scaffold and the unprocessed material using previously reported methods (Lee et al., 2011) ($n = 5$/scaffold type).

Cell Preparation and In Vitro Study

GFP-SC Preparation

Purified SC cultures were obtained from sciatic nerves of adult female Fischer rats (Envigo, Frederick, MD) following previously published protocols (Meijs et al., 2004). The resulting cultures were purified to greater than 95% (Takami et al., 2002). At passage 2 and at approximately 50% confluence, SCs were transduced in D10/mitogen medium overnight with a lentiviral vector encoding enhanced green fluorescent protein (GFP) at a multiplicity of infection of 30. D10 medium consisted of Dulbecco's modified Eagle's medium (DMEM, ThermoFisher Scientific, Carlsbad, CA) and 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, Logan, UT); the mitogens were pituitary extract (20 ug/mL, Biomedical Technologies S.L., Madrid, Spain), forskolin (2 uM, Sigma–Aldrich, St. Louis, MO), and heregulin (2.5 nM, Genentech,

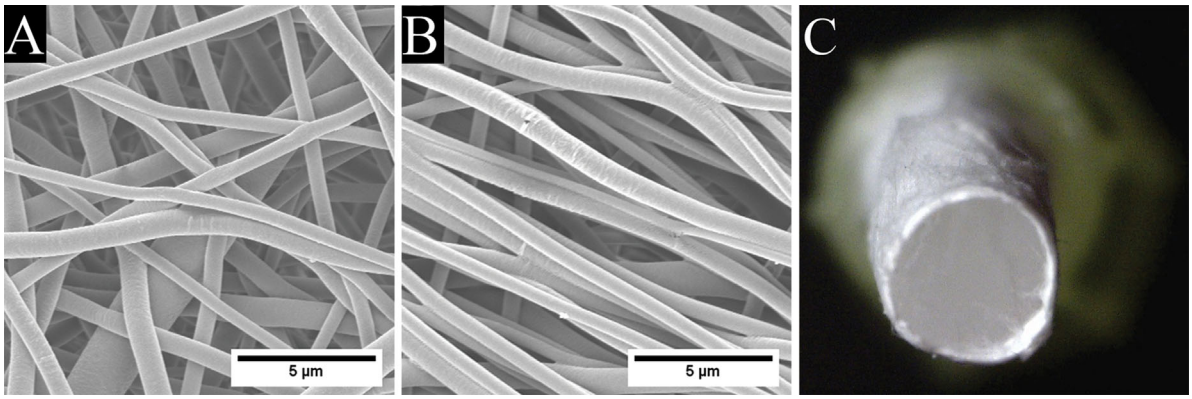

Figure 2. Scanning electron micrographs of random (**A**) and aligned (**B**) PVDF-TrFE fibrous scaffolds (10,000×). Cross-section of a hollow PVDF-TrFE conduit used for the in vivo studies (**C**).

San Francisco, CA). The production of the lentiviral vectors has been detailed elsewhere (Blits et al., 2005; Follenzi and Naldini, 2002). The transduction efficiency in the SC cultures was greater than 90%.

SC Culture

Polytetrafluoroethylene (PTFE) rings (ID, 5.94 mm; OD, 6.45 mm; 1.5 mm high; Zeus Inc., Orangeburg, SC) were placed on top of the aligned and random fibrous PVDF-TrFE disks in 96-well polypropylene plates to prevent them from folding and/or floating in the medium. Disks were either pre-conditioned with D10/ mitogen culture medium or coated with Matrigel (Corning Inc., Corning, NY) prior to cell seeding. Disks were preconditioned by incubating them in D10/mitogen medium for 24 h at 37°C. 2.5% (v/v) of Matrigel diluted in PBS was added to disks for 1 h then rinsed with PBS. GFP-SCs were seeded at a density of 5×10^4 cells/ cm^2 and culture medium was changed every 2–3 days.

Immunocytochemistry and Cell Viability Assay

GFP-SCs were fixed with 4% paraformaldehyde (PFA, Sigma–Aldrich) and their morphology assessed using confocal fluorescence microscopy (Clsi, Nikon, Japan) at days 1, 4, and 7. GFP-SC viability was measured at days 1, 4, and 7 using the ATP assay (CellTiter-Glo Luminescent Cell Viability Assay, Promega, Madison, WI). To perform this assay, GFP-SCs cultured on the disks were detached by scraping with pipette tips in culture medium. All cell suspensions were transferred to opaque-walled multi-well plates and CellTiter-Glo reagents were added to each well. The plate was incubated at room temperature for 10 min to allow the luminescent signal to stabilize and the luminescence was recorded using a plate reader (Flx800, BioTek Instrument Inc., Winooski, VT). An ATP standard curve was established by measuring the luminescence of a series of known ATP concentrations to determine the ATP concentrations in the samples. Cell viability was assessed based on the quantification of ATP. Four samples per group per time point were examined and studies were performed three times.

In Vivo Study: Complete Transection Model of SCI

Animals

Female adult Fischer rats (160–180 g body weight; Envigo) were housed according to NIH and USDA guidelines. The institutional Animal Care and Use Committee (IACUC) of the University of Miami approved all animal procedures. Animals were randomly assigned to receive random ($n = 7$) or aligned ($n = 7$) fibrous conduits. The animals were euthanized at 3 weeks post SC/conduit transplantation.

GFP-SC Collection and GFP-SC/Conduit Transplantation

Prior to transplantation, GFP-SCs were collected by trypsinization (0.05% trypsin; ThermoFisher Scientific; 0.05% EDTA for 5 min) and aliquots of 3×10^6 GFP-SCs were prepared, rinsed once with DMEM-F12 (ThermoFisher Scientific), and then centrifuged into a pellet. The pellets were kept on ice until transplantation.

Rats were anesthetized with an intraperitoneal injection of ketamine (Vedco Inc., St. Joseph, MO, 2.57 mg/100 g body weight) and xylazine (Lloyd Laboratories, Shenandoah Iowa, IA, 0.51 mg/100 g body weight). The back was shaved and aseptically prepared with chlorhexidine solution. Lacrilube opthalamic ointment (Allergen Pharmaceuticals, Irvine, CA) was applied to the eyes to prevent drying. During surgery, animals were kept on a heating pad to maintain body temperature at 37 ± 0.5°C. A multi-level laminectomy was performed from T7 to T9 (Fig. 1B) and all spinal roots were removed. The spinal cord was completely transected at T8 using angled microscissors. The stumps retracted and created a gap of approximately 2.5 mm (Fig. 1C). After hemostasis was achieved, the rostral stump was inserted into the 5 mm conduit followed by the insertion of the caudal stump into the other end with the pre-cut windows on the dorsal surface (Fig. 1D). 3×10^6 GFP-SCs were re-suspended in 10uL DMEM/F12 medium and then mixed with 10 uL Matrigel (Corning Inc.). The GFP-SC/Matrigel mixture was then injected into the conduit through one of the pre-cut windows using a

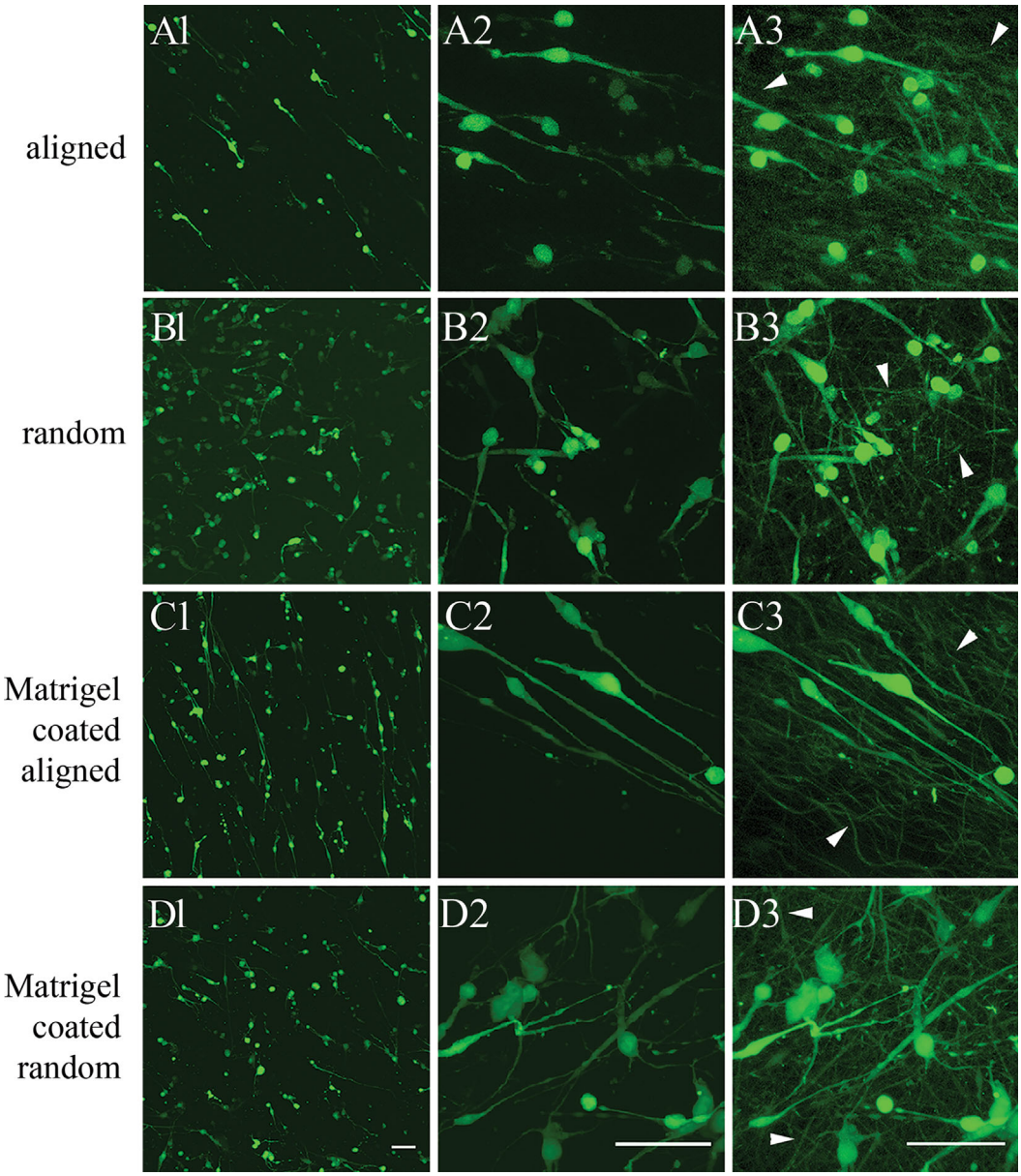

Figure 3. Confocal microscope images of GFP-SCs on PVDF-TrFE aligned (**A**), random (**B**), Matrigel-coated aligned (**C**), and Matrigel-coated random (**D**) fibrous scaffolds at day 7. Fibers of the scaffold are observed to be autofluorescent (green, arrowheads) in A3-D3. A1-D1, 20×; A2-D2; and A3-D3, 60×. Scale bars = 50 μm.

micropipettor. The flap of the window was pushed to close the windows (Fig. 1E). The muscles were sutured and the skin was closed with clips. Immediately after surgery, rats received a subcutaneous injection of 10 mL lactated Ringers (LR) solution. The rats were kept in warm cages with food and water readily available. Buprenorphin (0.3 mg/1 mL, Buprenex, Reckitt Benckiser Healthcare Ltd., Berkshire, UK) was administrated twice a day for 3 days immediately after surgery to reduce pain. Gentamycin (APP Pharmaceuticals, LLC, Schaumburg, IL, 40 mg/mL) was administrated once a day for 7 days starting immediately after surgery to reduce infection. Bladders were emptied manually twice a day until bladder function returned.

Tissue Preparation and Immunohistochemistry

Three weeks after conduit/SC transplantation, rats were terminally anesthetized with ketamine (Vedco Inc., 60 mg/kg) and xylazine (Lloyd Laboratories, 12 mg/kg) and transcardially perfused with chilled heparinized saline (0.9% NaCl, pH 7.4) followed by ice-cold 4% PFA in PBS (pH 7.4). Tissue was left in 4% PFA overnight before the spinal cord was dissected for all tissue preparation. Cords prepared for immunostaining were cryoprotected with 30% sucrose in PBS overnight. Segments of the spinal cord centered at the SC/conduit were removed and embedded in gelatin (12% in PBS). The tissue segments were cut into 20 μm sagittal sections using a

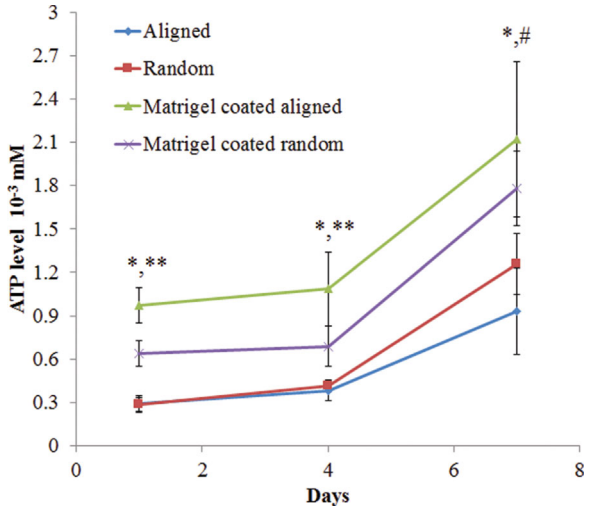

Figure 4. ATP levels for SCs cultured on PVDF-TrFE scaffolds. ATP levels for SCs on Matrigel-coated scaffolds were significantly higher than uncoated scaffolds at days 1, 4, and 7 ($^{*}P < 0.05$). ATP levels for SCs at day 7 were significantly higher than at days 1 and 4 for uncoated and Matrigel-coated scaffolds ($\#P < 0.05$). ATP levels for SCs on Matrigel-coated aligned fibrous scaffolds were significantly higher than Matrigel-coated random fibrous scaffolds at days 1 and 4 ($^{**}P < 0.05$).

cryostat ($n = 5$/group) and stored at $-20°C$ until processed. In separate animals, a 1 mm transverse slice was removed from the center of the SC/conduit and prepared for 1 μm plastic sectioning as previously described (Bates et al., 2011). The sections were then stained with toluidine blue for morphological evaluation.

For immunostaining, the sections were thawed and rinsed with PBS and blocked with blocking solution, 10% normal goat serum (NGS) and 0.1% triton-x in PBS, for 1 h at room temperature followed by incubation with primary antibodies in blocking solution overnight at 4°C. The sections were stained with primary antibodies against DβH (dopamine β hydroxylase, 1:500, mouse, EMD Millipore, Billerica, MA), RECA-1 (endothelial cell, 1:700, mouse, AbD Serotec, Kidlington, UK), GFAP (glial fibrillary acidic protein, 1:500, rabbit, Dako, Carpinteria CA, or mouse, Covance, Princeton, NJ), NF (neurofilament, 1:500, rabbit, EnCor Biotechnology Inc., Gainesville, FL), CGRP (calcitonin gene related peptide, 1:800, rabbit, EMD Millipore), or GFP (green fluorescent protein, 1:1000, chicken, EMD Millipore). Sections were then rinsed with PBS and incubated with Hoechst 355 (1:800, nuclear stain) and secondary antibodies (1:200, all from ThermoFisher Scientific, goat anti-mouse IgG-546 or 660, goat anti-rabbit IgG-546 or 660, or goat anti-chicken IgY-488) with 1% NGS in PBS for 1 h at room temperature. Sections were coverslipped with Vectamount fluorescence mounting medium (Vector Laboratories, Burlingame, CA) and sealed with CoverGrip Coverslip sealant (Biotium, Hayward, CA).

Quantification of Axon Regeneration and Extension of GFAP$^+$ Astrocyte Processes

The numbers of regenerated DβH$^+$ axons and of GFAP$^+$ astrocyte processes were counted by a line-transect method as previously described on parasagittal sections (Fig. 1F) (Williams et al., 2012, 2015). Briefly, the 0 line was defined as the location where dense expression of GFAP$^+$ astrocyte somata terminated at the rostral cord/SC bridge interface on the tissue section that contained the rostral spinal cord inserted farthest into the conduit for each animal. This 0 line marked the start of the rostral cord/SC bridge interface. The distance between the 0 line and the end of the conduit in this section was used to establish the location of the 0 line in other sections of the same animal. Dorsoventral lines were drawn on the tissue (visualized by Hoechst staining) within the conduit, and measured at 0.25, 0.5, 1, 1.5, 2, and 2.5 mm beyond the 0 line. The numbers of immunostained GFAP$^+$ astrocyte processes and DβH$^+$ axons that crossed the transverse plane at each dorsoventral line were counted. The area of the transverse plane of tissue at each distance was determined by multiplying the tissue section thickness (20 μm) by the length of the dorsoventral line. Therefore, the results were reported as number of axons/mm^2. The numbers of DβH$^+$ axons in both random and aligned conduits at each dorsoventral line were further separated into groups with more than or less than 200 GFAP$^+$ astrocyte processes.

Statistical Analyses

All in vitro data were analyzed using two-way ANOVA and Tukey Pairwise Comparisons with 95% confidence (Minitab v17.1.0, Minitab Inc., State College, PA). All in vivo data were analyzed using two-way ANOVA (multivariate general linear model in SPSS v22, IBM Corp., Armonk NY) with LSD (Fisher's least significant difference) post-hoc tests. Significance was determined at $P < 0.05$. All results were expressed as mean ± standard deviation.

Results

Scaffold Morphology and Characterization

The fibers of the PVDF-TrFE scaffolds were uniform in morphology as observed by SEM (Fig. 2). The average fiber diameter for aligned scaffolds was 548 ± 139 nm and 711 ± 222 nm for random scaffolds. No statistical differences were detected between groups for fiber diameters. The inter-fiber spacing for aligned scaffolds, 4.13 ± 1.27 μm, was significantly less than random scaffolds, 7.03 ± 1.91 μm ($P < 0.05$). The degree of alignment was 89.3% ± 9.9% for aligned scaffolds. The porosity was 87.1 ± 1.5% and 88.0 ± 1.1% for aligned and random scaffolds, respectively. No statistical differences in porosity were found.

In Vitro Study

GFP-SCs attached well to both aligned and random scaffolds (Fig. 3) and were extended along the fibers on both Matrigel-coated (Fig. 3C and D) and uncoated (Fig. 3A and B) scaffolds. GFP-SCs attached parallel to each other, in the direction of the fibers, on both uncoated (Fig. 3A3) and Matrigel-coated (Fig. 3C3) aligned scaffolds. In contrast, GFP-SCs were attached in an unorganized manner on uncoated (Fig. 3B3) and Matrigel-coated (Fig. 3D3) random scaffolds. ATP levels increased significantly from days 4 to 7 (Fig. 4) when SCs were cultured on Matrigel-coated and uncoated scaffolds. No statistical differences were detected between uncoated aligned and random scaffolds over time. At days 1 and 4, ATP levels were higher for SCs seeded on Matrigel-coated aligned than on

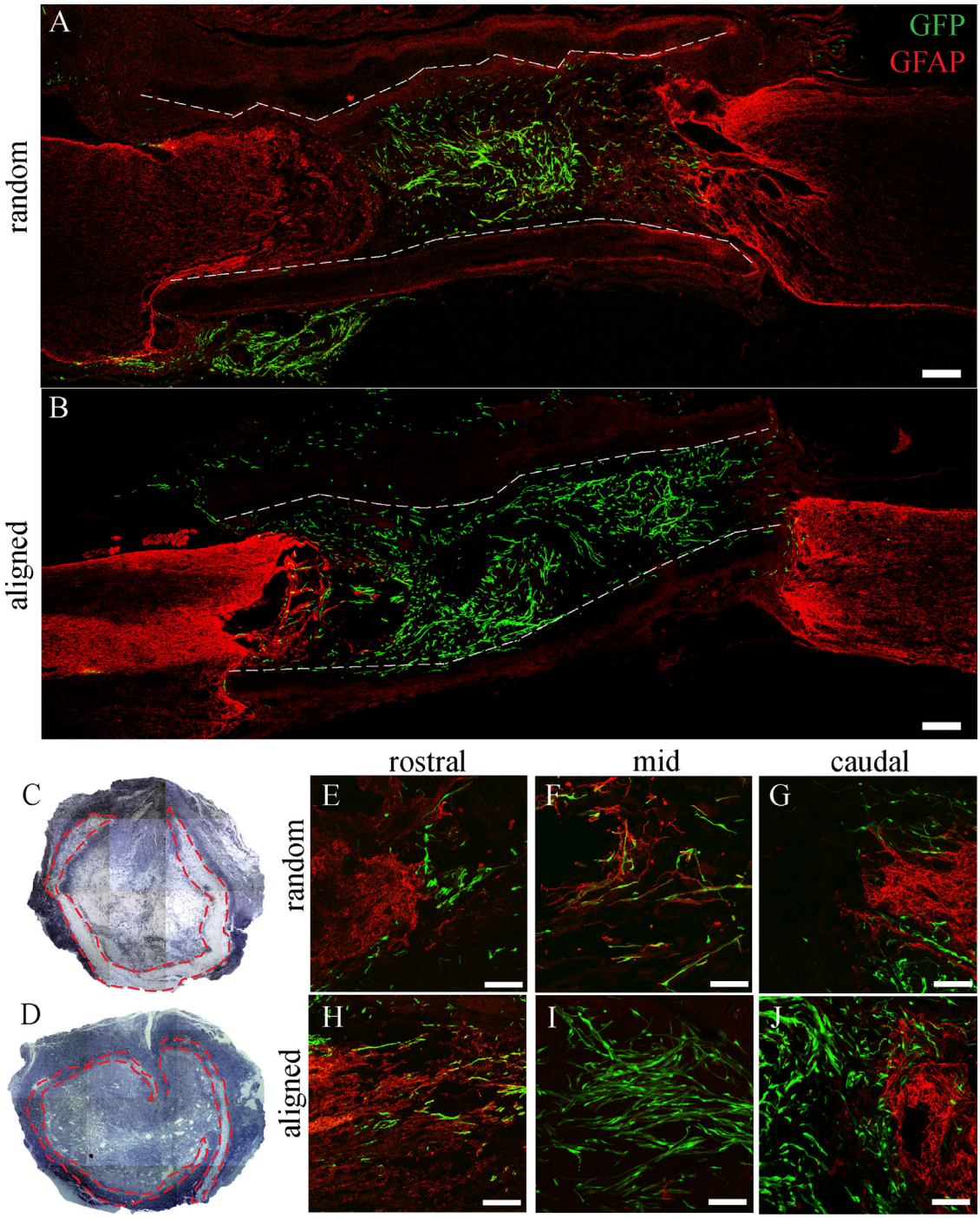

Figure 5. Confocal fluorescence images of sagittal sections of spinal cords transplanted with GFP-SCs in random (**A**) and aligned (**B**) PVDF-TrFE conduits (outlined by white dashed lines). GFP^+ SC survival was observed in both types of conduits (**A**,**B**). Denser tissue was seen in the aligned (**D**) compared to the random (**C**) conduits in 1 μm plastic sections (conduits outlined by red dashed lines). Confocal images illustrate rostral interfaces (**E**,**H**, near the 0 line, Fig. 1F), caudal interfaces (**G**,**J**, beyond 2.5 mm, Fig. 1F), and the middle of the transplant (**F**,**I**) in both types of conduits. Scale bars: A and B, 200 μm; E–J, = 100 μm.

Matrigel-coated random scaffolds. At all time points, higher ATP levels were detected for SCs on Matrigel-coated than on uncoated scaffolds. The results demonstrated that both random and aligned scaffolds, with or without Matrigel coating, supported SC attachment and viability.

In Vivo Study

GFP-SCs were observed throughout both random (Fig. 5A) and aligned (Fig. 5B) conduits three weeks post transplantation but were distributed more uniformly along the lumen of the aligned

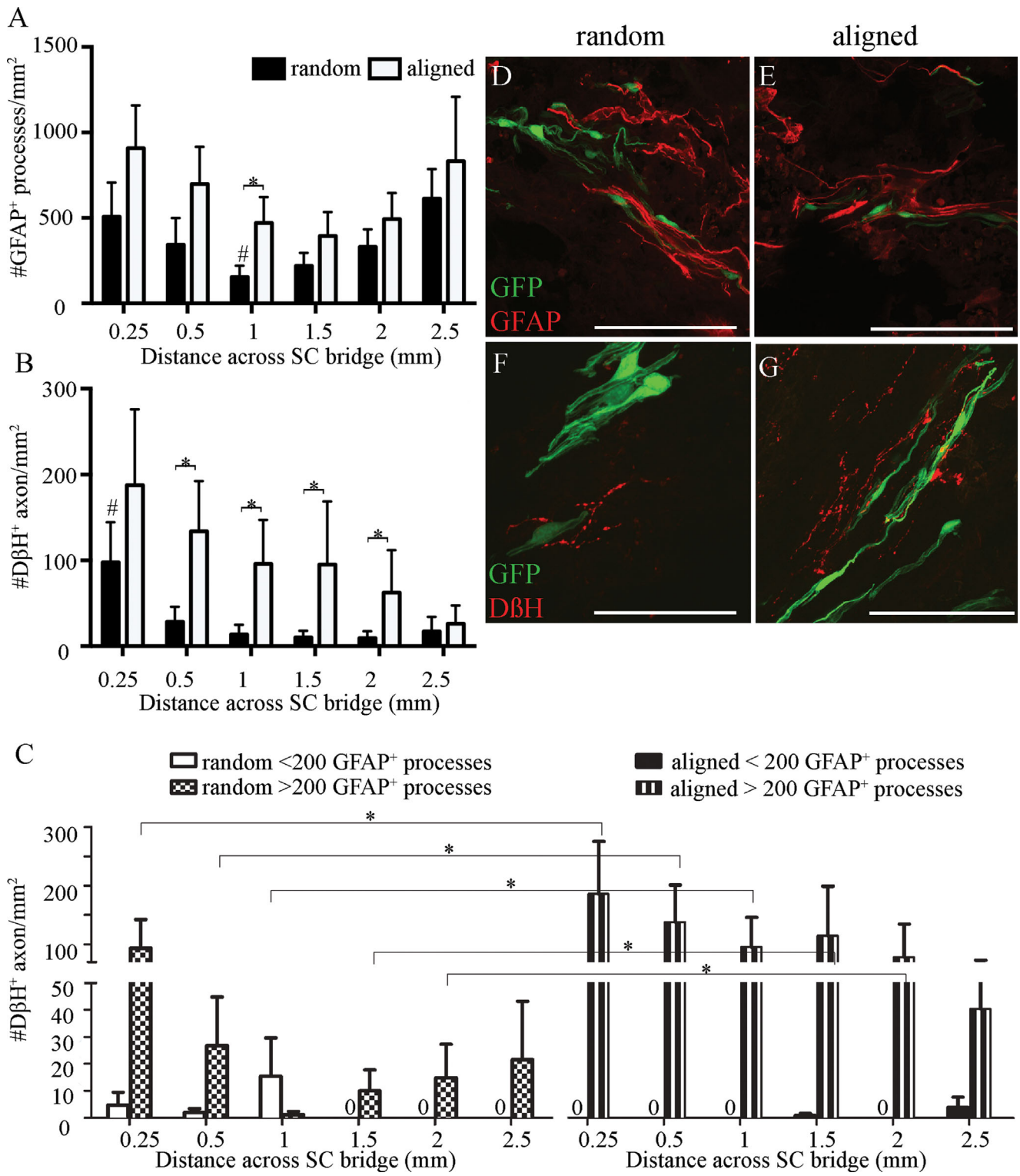

Figure 6. Quantification of immunostained GFAP$^+$ processes (**A**) and DβH$^+$ axons (**B**) at various distances from the rostral interface using the line-transect method (Fig. 1F). At 1 mm, significantly more GFAP$^+$ processes were found in aligned fibrous conduits than in random fibrous conduits (A, $^*P < 0.05$). Significantly fewer GFAP$^+$ processes were at 1 mm than at 0.25 and 2.5 mm from the rostral interface in random conduits (A, $\#P < 0.05$). Significantly more DβH$^+$ axons were counted in aligned than in random fibrous conduits at 0.5–2 mm (B, $^*P < 0.05$). Significantly more DβH$^+$ axons populated random fibrous conduits at 0.25 mm than at all other distances (B, $\#P < 0.05$). The numbers of DβH$^+$ axons in random versus aligned fibrous conduits when comparing SC implants containing less or more than 200 GFAP$^+$ processes (**C**). There were significantly more DβH$^+$ axons in aligned conduits with more than 200 GFAP$^+$ processes than in random conduits at all respective distances except at 2.5 mm (C, $^*P < 0.05$). Close association of GFAP$^+$ processes and GFP-SCs at the rostral interface of random (**D**) and aligned (**E**) fibrous conduits was observed in confocal fluorescence images. DβH$^+$ axons also co-localized with GFP-SCs in random (**F**) and aligned (**G**) fibrous conduits at 0.25 mm caudal to the rostral interface in confocal fluorescence images. Scale bars: D–G, 50 μm.

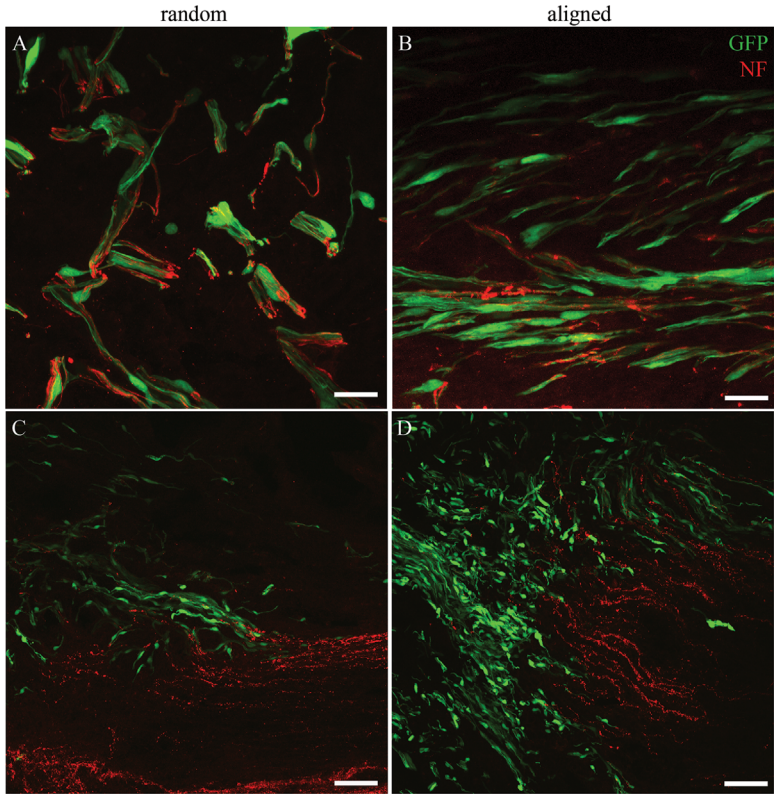

Figure 7. Confocal fluorescence images of GFP-SCs in the center of the graft (**A**,**B**) or toward the caudal interface (**C**,**D**) in random (**A**,**C**) and aligned (**B**,**D**) conduits. The GFP-SCs and NF^+ axons were found parallel along the longitudinal axis of the aligned conduit (**B**) in contrast to the less organized arrangement in the random conduit (**A**). More GFP-SCs were observed toward the caudal interface in aligned (**D**) than in random (**C**) conduits. $CGRP^+$ axons were found to be more abundant entering the caudal interface of the aligned (**D**) than in random (**C**) conduits. Scale bars: A–D, 50 μm.

conduits, indicative of a higher cellular content. SCs were more parallel along the rostral-caudal axis in the aligned (Fig. 5I) than in the random (Fig. 5F) conduits. In 1 μm cross sections, tissue density was observed to be higher in the aligned (Fig. 5D) than in the random (Fig. 5C) conduits. No excessive scar/fibrotic tissue was observed around the conduit exterior (Fig. 5A–D). In general, $GFAP^+$ borders at the rostral (Fig. 5E and H) or caudal (Fig. 5G and J) interfaces between the spinal cord and SC bridge were similar in both types of conduits. Interdigitated astrocyte processes and SCs created irregular borders at both interfaces in both types of conduits (Fig. 5E, G, H, and J).

Significantly more $GFAP^+$ astrocyte processes were found at 1 mm from the rostral interface in the aligned than in the random conduits (Fig. 6A, $P < 0.05$). Among random conduits, fewer $GFAP^+$ processes were counted at 1 mm than at 0.25 and 2.5 mm from the rostral interface (Fig. 6A, $P < 0.05$). More $D\beta H^+$ axons were observed in aligned conduits at 0.5–2 mm from the rostral cord/SC bridge interface (Fig. 6B, $P < 0.05$). The number of $D\beta H^+$ axons in random conduits was highest at 0.25 mm compared to other distances (Fig. 6B, $P < 0.05$). There were no $D\beta H^+$ axons in aligned conduits with less than 200 $GFAP^+$ processes at all distances except at 1.5 and 2.5 mm (Fig. 6C). In random conduits, there were no $D\beta H^+$ axons at distances of 1.5 mm or more when there were less than 200 $GFAP^+$ processes (Fig. 6C). The number of $D\beta H^+$ axons in aligned conduits with more than 200 $GFAP^+$ processes was significantly greater than in random conduits at all respective distances except at 2.5 mm (Fig. 6C, $P < 0.05$). Close association of $GFAP^+$ processes and GFP-SCs at the rostral interface of random (Fig. 6D) and aligned (Fig. 6E) conduits was observed. No GFP-SCs were observed to stain positive for GFAP. In vitro, the absence of axon contact induces a cytoskeletal cellular response in SCs to express higher levels of GFAP (Bianchini et al., 1992). In vivo, SCs express GFAP but the level is too low to be detectable by immunostaining. $D\beta H^+$ axon co-localization with GFP-SCs also was observed in both random (Fig. 6F) and aligned (Fig. 6G) conduits at 0.25 mm caudal to the rostral interface. In sum, more $GFAP^+$ processes and $D\beta H^+$ axons were found in aligned than in random conduits; $D\beta H^+$ axon growth into the SC bridge improved with higher numbers of $GFAP^+$ processes. SCs were closely associated with $GFAP^+$ processes and $D\beta H^+$ axons.

Close association of GFP-SCs with NF^+ axons also was observed. GFP-SCs and axons were parallel to the longitudinal axis of the aligned conduits (Fig. 7B) in contrast to the random ones (Fig. 7A). Sensory $CGRP^+$ axons were closely associated with GFP-SCs, entering the SC bridge from the caudal interfaces of both random (Fig. 7C) and aligned (Fig. 7D) conduits.

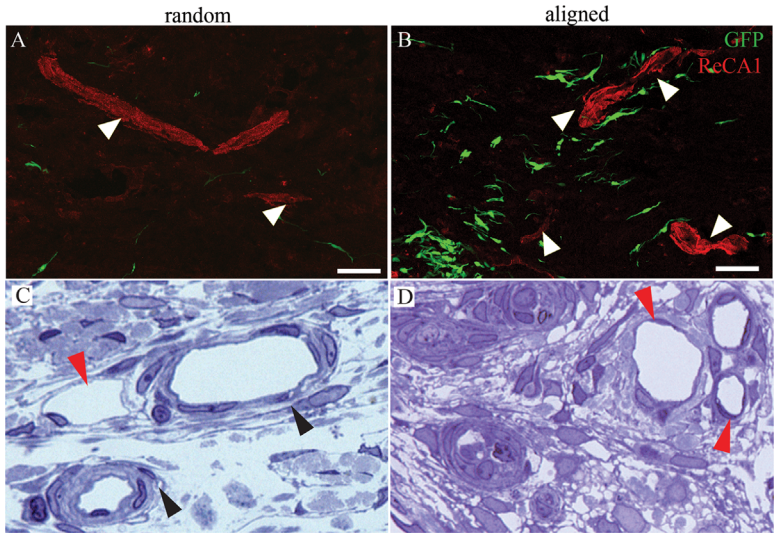

Figure 8. Blood vessels in the mid-bridge regions, immunostained with anti-ReCA1 antibody, were observed in both random (**A**) and aligned (**B**) conduits (white arrowheads) (confocal fluorescence images, scale bars: 50 μm). Venules (red arrowheads) and arterioles (black arrowheads) vessels were observed in the mid-bridge region in random (**C**) and aligned (**D**) conduits (1 μm plastic sections, toluidine blue stain, 60×).

Blood vessels were found in the center of the SC bridge in both random and aligned conduits following immunostaining for ReCA1 (Fig. 8A and B, respectively). They were also identified morphologically in transverse plastic sections (Fig. 8C and D, respectively). Both arteriole (black arrowheads, thicker-walled) and venule (red arrowheads, thinner-walled) vessels were observed.

Discussion

This is the first and initial study to investigate the feasibility of employing an electrospun PVDF-TrFE conduit with SCs as a transplant to repair the spinal cord after a complete transection injury. The complete transection model was chosen because it is the most rigorous method to evaluate axon regeneration compared to a contusion or hemi-section model where there is some retention of tissue around the injury. In the complete transection model, where there is no remaining bridge of tissue around the injury site, axon growth across a spinal cord/SC bridge interface is clearly regenerative growth rather than axon sparing or sprouting. Also, the interfaces between the completely transected host spinal cord and the SC transplants are easier to evaluate compared to other lesion models. We are able to identify whether the interface is inhibitory or permissive to axon growth. Electrospun PVDF-TrFE conduits were chosen because they are not only biocompatible but also have the potential to improve axon growth after SCI based on earlier work (summarized by Rajabi et al., 2015). Here we report that they supported (i) SC survival, (ii) regeneration of sensory ($CGRP^+$) and brainstem ($D\beta H^+$) axons across the spinal cord/SC bridge interface into the conduit, (iii) extension of astrocyte/$GFAP^+$ processes into the SC bridge, and (iv) blood vessel formation in the SC bridge. SCs and NF^+ axons extended along the conduit, parallel to the rostral-caudal axis, corresponding to the direction of the aligned fibers of the conduit. More $D\beta H^+$ axons and $GFAP^+$ processes were observed in aligned than random conduits. No behavioral assessments were performed in this study because the animals were kept for a short period after transplantation and would not have shown substantial improvement.

PVDF-TrFE aligned and random scaffolds supported SC attachment and viability in vitro. SC viability increased over time on Matrigel-coated and uncoated scaffolds as indicated by increasing ATP levels. Higher cell viability was observed on Matrigel-coated compared to uncoated scaffolds over time. The main components of Matrigel, laminin and type IV collagen, are basement membrane proteins that enable cell adhesion. Thus, the presence of these basement membrane proteins on PVDF-TrFE may allow for better cellular attachment and improved viability. Interestingly, SCs on Matrigel-coated aligned scaffolds exhibited greater cell viability at early time points than Matrigel-coated random scaffolds, suggesting the physical cue of fiber alignment when Matrigel is present also contributes to SC viability. Therefore, in vitro findings supported the use of Matrigel as a cell carrier with the PVDF-TrFE conduit to improve SC survival after transplantation.

Both random and aligned conduits supported SC survival in vivo. The interior alignment of the aligned conduit contributed to a more uniform distribution of transplanted SCs and a denser tissue bridge than the random conduits. This observation is similar to other studies where aligned fibers promote cell migration (Dickinson et al., 1994; Petrie et al., 2009; Sundararaghavan et al., 2013) and neurite extension (Kijenska et al., 2012; Liu et al., 2010; Yang et al., 2004). In other studies, spinal axons (Yao et al., 2011), hippocampal neurons (Yao et al., 2008), and embryonic stem cell-derived neural cells (Li et al., 2014) showed directed migration when electric fields were applied. Electric charge generated in the PVDF-TrFE conduits may come from minute mechanical deformations in the SC bridge due to random body movement, spinal cord deformation, and/or cell attachment or migration. Piezoelectric guidance channels used

in peripheral nerve repair have demonstrated greater numbers of myelinated axons than in non-piezoelectric controls where deformations in the implanted limb were found to induce significant luminal charges (Aebischer et al., 1987b; Fine et al., 1991). More recent studies suggest that the location of the piezoelectric implant in the body can affect the biological response due to the extent of anatomical deformation and random animal movements (Guo et al., 2012). Future studies will measure the piezoelectric activity of the PVDF-TrFE conduit in situ to understand if and how piezoelectricity may contribute to transplanted SC distribution and axon regeneration.

In order to facilitate repair after SCI, the regenerating axons must be able to cross both rostral and caudal interfaces between the spinal cord and the SC transplant. When the GFAP antibody-immunostained interface between the host spinal cord and transplant is sharply defined, it is not as permissive for axon crossing as an interface that is irregular, due to the interdigitation of the astrocyte processes and transplanted SCs (Williams et al., 2015). Astrocytes are permissive for axon growth when they elongate as linearly oriented processes into the SC transplant. Mechanisms that influence the formation of a distinct rather than an irregular interface (where astrocyte processes extend into the cell transplant) are not yet fully known. In situ gelling of Matrigel after SC transplantation (Williams et al., 2015), providing glial-derived neurotrophic factor along with SC transplantation (Deng et al., 2011, 2013; Iannotti et al., 2003), and pre-treatment of glial-restricted precursors with bone morphogenetic protein (Davies et al., 2006) all favor astrocyte extension into cellular grafts.

Our earlier findings were observed inside PAN/PVC conduits with SCs in pre-gelled Matrigel (Xu et al., 1995a,b, 1997, 1999) or in situ-gelled Matrigel (Williams et al., 2015). The use of PVDF-TrFE conduits in the present study resulted in similar yet improved results. In both types of PVDF-TrFE conduits, irregular spinal cord/SC bridge interfaces with more intermingling of $GFAP^+$ astrocyte processes with GFP^+ SCs were observed. PVDF-TrFE chemistry and/or piezoelectric properties of the conduits may have contributed to the formation of a more permissive rostral interface because more astrocyte processes crossed the rostral interfaces compared to the previous study using a non-piezoelectric PAN/PVC conduit (Williams et al., 2015). In addition, more $D\beta H^+$ axons were counted crossing the rostral interfaces in both types of PVDF-TrFE conduits compared to the PAN/PVC conduits (Williams et al., 2015). More $D\beta H^+$ noradrenergic axons regenerated across the SC bridge when greater numbers of $GFAP^+$ astrocyte processes were present, especially in the aligned conduits. Elongated SCs were co-localized with astrocyte processes (Fig. 6D and E) and brainstem $D\beta H^+$ axons (Fig. 6F and G) in the SC bridge as in the previous study (Williams et al., 2015). Significantly more $D\beta H^+$ noradrenergic axons entered aligned than random conduits, tapering off in number with increasing distance from the rostral interface.

Specific axon populations, $D\beta H^+$ and $CGRP^+$ axons, were visualized in this initial study of the PVDF-TrFE conduits. In the uninjured spinal cord, descending noradrenergic ($D\beta H^+$) axons from the brainstem project throughout the spinal cord and participate in pain modulation and autonomic and motor responses (Lindvall et al., 1983). The finding that these brainstem axons regenerated from the rostral interface into the Matrigel/SC bridge with no additional interventions also shows the promise of these conduits to transplant SCs after SCI. Ascending sensory $CGRP^+$ axons were observed to enter the SC bridge from the caudal interface in both random and aligned conduits; the aligned conduits appeared to contain more $CGRP^+$ axons. These axons, nociceptive in type, are located in the dorsal horn of the normal spinal cord (Xu et al., 1999). The presence of more $CGRP^+$ axons in aligned conduits suggests that these conduits could aid in promoting sensory recovery. We submit that a combination of fiber alignment and PVDF-TrFE, which has piezoelectric properties, contributed to our observations. These conduits, therefore, deserve further characterization, including investigation of their piezoelectricity in vivo and maintaining experimental animals for longer periods to more fully assess SC and axon responses.

Conclusions

This is the first study to demonstrate the potential of using electrospun PVDF-TrFE aligned fibrous conduits with SCs for spinal cord repair. SC viability in vitro and survival in vivo were shown in this feasibility study. In vitro studies also indicated that Matrigel coating improved SC attachment. Interdigitated host astrocyte and SC bridge interfaces were observed in animals receiving both types of conduits, indicating a permissive interface for axon regeneration. Aligned conduits promoted more extension of astrocyte processes from the rostral cord with accompanying descending $D\beta H^+$ axon regeneration along the conduit and farther into the SC bridge compared to random conduits. Sensory axons were present at the caudal interface in both types of conduits. They appeared to be more numerous in the aligned conduits and need quantitative confirmation in follow up studies. Future studies will further characterize the regenerative responses and the piezoelectric activity of these conduits under in vivo conditions. Future longer-term in vivo studies will include behavioral analysis and intra-spinal retrograde and anterograde tracing to evaluate both ascending and descending axon regeneration through the bridge/conduit. Growth factors and drugs also could be incorporated into the fibrous conduits for sustained release for additional therapeutic benefit.

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Schwann Cell loaded PVDF-TrFE Scaffolds Promote Axon Regeneration after Spinal Cord Injury

Yee-Shuan Lee[1*], Siliang Wu[2*], Treena Livingston Arinzeh[3], Mary Bartlett Bunge[1]
[*]contributed equally to the work, [1]The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami FL 33101, [2]Department of Material Science and Engineering, [3]Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, NJ 07102.

Introduction: Schwann cell (SC) transplantation has been utilized for spinal cord repair and demonstrated to be a promising therapeutic strategy. In this study, we investigated polyvinylindine trifluoroethylene (PVDF-TrFE) scaffolds, consisting of aligned fibers, to promote SC supported axon regeneration both *in vitro* and *in vivo*. Previous studies showed that PVDF-TrFE fibrous scaffolds supported SC attachment and dorsal root ganglia (DRG) neurite extension (Lee 2011; Wu 2016). Matrigel™ has been routinely used to improve SC attachment and axonal growth but it will not be appropriate for clinical applications because Matrigel is derived from cancer cells. In this study, Matrigel coated and uncoated PVDF-TrFE fibrous scaffolds were compared for promoting neurite extension in SC-DRG co-cultures *in vitro* and in a complete transection spinal cord rat model *in vivo*. Our overall goal was to determine if PVDF-TrFE scaffolds in combination with SCs could support neurite extension without the use of Matrigel.

Materials and Methods: PVDF-TrFE was electrospun to form aligned fibrous scaffolds as previously described (Lee 2011; Wu 2016). *In vitro:* SCs expressing green fluorescent protein (GFP-SCs) were seeded onto Matrigel coated or uncoated PVDF-TrFE scaffolds at 6,000 and 15,000 cells/scaffold followed by seeding E17 rat DRGs at day 4. SC-DRG co-cultures were fixed and stained for neurofilaments at day 6. Neurite extension was measured by image analysis. *In vivo*: Laminectomy was performed on adult Fischer rats followed by complete transection at T8. PVDF-TrFE conduits containing aligned fibrous layers were coated with (group MM) or without (group DD) Matrigel and seeded with $3x10^6$ GFP-SCs prior to transplantation. During transplantation, $3x10^6$ GFP-SCs were transplanted with Matrigel between the layers in MM. DD were transplanted with $3x10^6$ GFP-SCs SCs without Matrigel between the layers. *Tissue analysis:* The rats were perfused at 6 wks; cryostat 20µm horizontal sections were stained with antibodies against GFP and RT97 (general axon). Plastic section was prepared to quantify myelinated and unmyelinated axons.

Results and Discussion: SCs promoted greater neurite extension in the SC-DRG co-cultures compared to DRG only cultures on uncoated scaffolds (Fig 1). Although Matrigel coating with or without SCs induced robust neurite outgrowth, no statistical difference in neurite length was detected between Matrigel coated and uncoated scaffolds for high SC seeding density (15K). I*n vivo*, no significant difference was observed in the number of myelinated and unmyelinated axons (Fig 2) with the presence of Matrigel. More GFP-SCs were observed with Matrigel coating (MM, Fig 2) in the center of the conduit but a similar number of $RT97^+$ axons was observed in both groups and throughout the conduit.

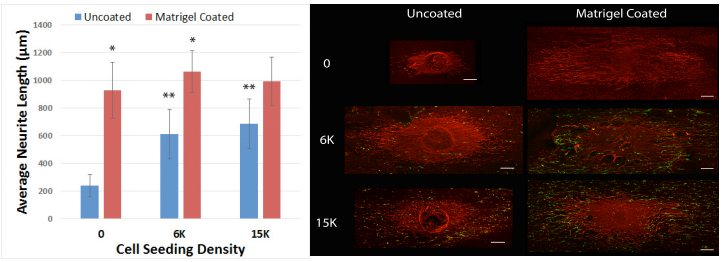

Figure 1. Neurite extension of DRG and SC-DRG co-cultures for low (6K) and high (15K) SC seeding densities on Matrigel coated and uncoated PVDF-TrFE scaffolds. *p<0.05, Matrigel coated have significantly longer neurites than on uncoated groups. **p<0.05, low (6K) and high (15K) SC seeding densities have significantly longer neurites than no (0K) SCs on uncoated scaffolds. Scale bar= 200µm.

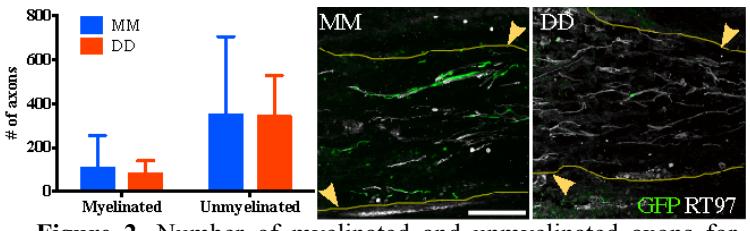

Figure 2. Number of myelinated and unmyelinated axons for groups MM and DD. Confocal fluorescent images of GFP-SCs and $RT97^+$ axons in the center of MM and DD conduits. Layers are outlined by the solid line indicated by the arrowheads. Scale bar=50um.

Conclusions: PVDF-TrFE scaffolds have the potential to provide a more clinical appropriate approach to achieve axon regeneration in spinal cord injury repair without the use of Matrigel.

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Schwann Cell--laden PVDF-TrFE Conduits for Spinal Cord Repair

Affiliations:

1. The Miami Project to Cure Paralysis, University of Miami, Miami, Florida, United States
2. Department of Material Science and Engineering, New Jersey Institute of Technology, Newark, Florida, United States
3. Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, New Jersey, United States
4. Department of Cell Biology, University of Miami, Miami, Florida, United States

Authors:

Yee-Shuan Lee[1]
Siliang Wu[2]
Treena L Arinzeh[3]
Mary B Bunge[1,4]

Disclosures:

Required disclosures are complete

Presenter:

Yee-Shuan Lee

Body:

Introduction

The goal in this study was to develop a piezoelectric polyvinylidenefluoride-trifluoroethylene (PVDF-TrFE) conduit containing Schwann cells (SCs) for spinal cord repair. Piezoelectric materials can generate electrical activity with minute deformation. PVDF-TrFE aligned fibers facilitate axon extension by contact guidance in vitro [1][2]. This study first evaluated the conduit design where PVDF-TrFE aligned fibers were fabricated into hollow tubes or tubes filled with aligned fibers. Then, fiber filled conduits were either coated with or without Matrigel and loaded with SCs and implanted into a transected spinal cord. We hypothesized that the fibers filled within the tubes would facilitate SC survival without Matrigel and direct axon growth. Eliminating Matrigel, derived from mouse sarcoma cells, will make this approach more clinically relevant.

Materials and Methods

Fabrication: PVDF-TrFE aligned fibers were fabricated via electrospinning. Conduits were formed as hollow tubes or tubes filled with aligned fibers (filled tubes) (Exp1). Filled tubes were pre-coated with or without Matrigel overnight and seeded with 3 x 106 GFP-SCs and incubated for 2h prior to transplantation (Exp2). See Table 1 for list of experimental groups. Transplantation: Laminectomy was performed on adult Fischer rats followed by complete transection at T8. The hollow (Exp1) or filled (Exp1&2) tubes was inserted between the stumps and GFP-SCs in DMEM with or without Matrigel were injected into the tubes. Tissue analysis: The rats were perfused at 3 (Exp1) or 6 (Exp2) wks; cryostat 20μm sagittal sections were stained with antibodies against GFP, GFAP (astrocyte), RT97 (general axon), and CGRP (Calcitonin gene-related peptide, sensory neuron).

Table 1. Experimental groups of Exp 1 and 2.

Experiment 1 (Exp 1)

Groups	SCs/Matrigel injected into tube at time of transplantation into spinal cord	SCs seeded without Matrigel 2h before transplantation into spinal cord
Hollow	+	-
Filled	-	+

Experiment 2 (Exp 2)

Groups	Filled tube precoated with Matrigel prior to SC seeding without Matrigel 2h before transplantation into spinal cord	SC/Matrigel injection into filled tubes at time of transplantation into spinal cord
MM	+	+
MD	+	-
DM	-	+
DD	-	-

Results and Discussion

Exp1 (Fig 1): More SCs appeared to be in hollow than in filled tubes possibly due to (1) SC survival supported by the Matrigel and (2) less space within the lumen of filled tubes. RT97+ axons were associated with SCs in both tubes and along the rostral/caudal axis on the inner fibers suggesting directed axon growth. CGRP+ sensory axons were observed only at the caudal host-SC interface in hollow tubes but were closely associated with SCs along the caudal/rostral axis in filled tubes.

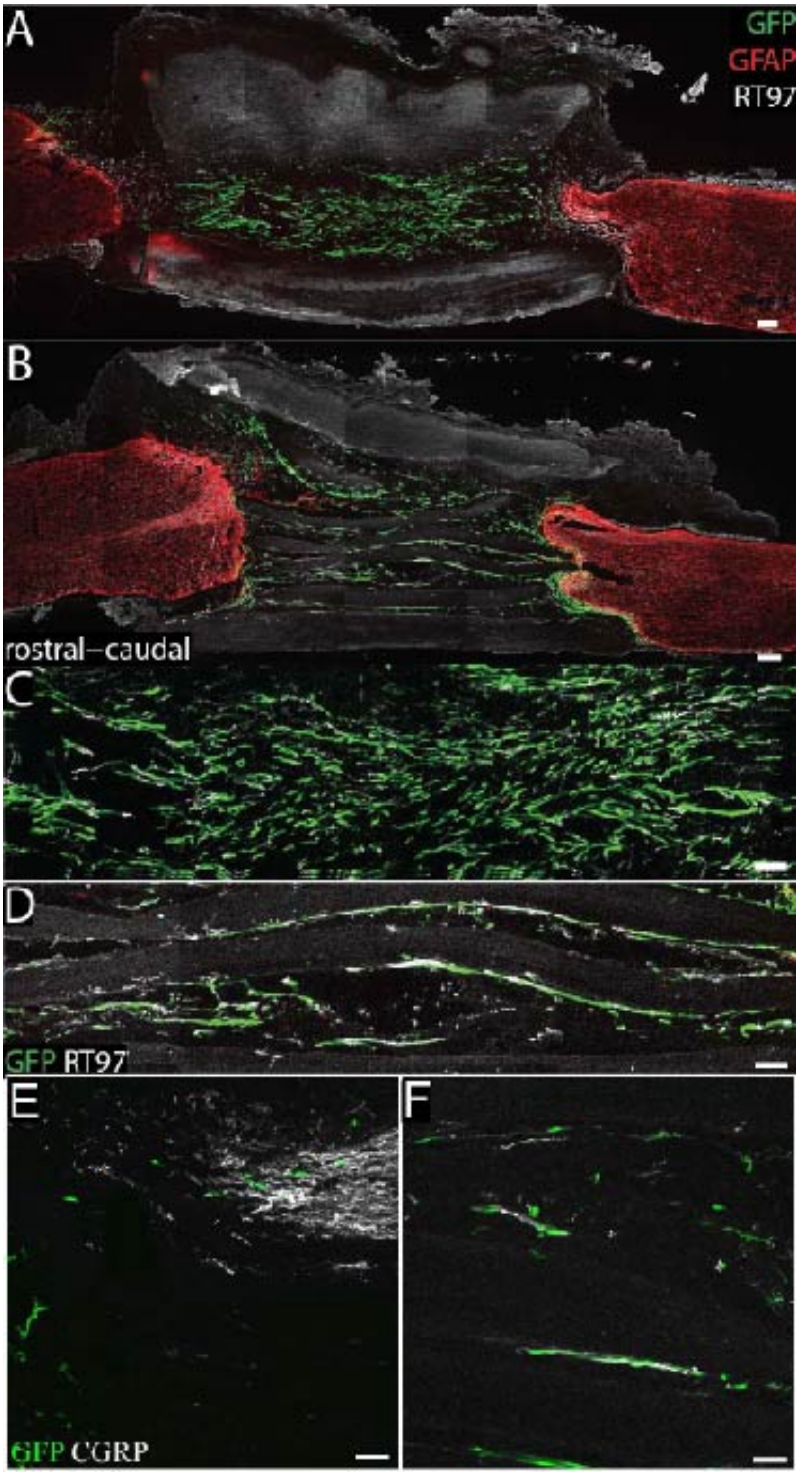

Fig 1. (Exp 1) Confocal fluorescent images of: SC bridges 3 wks post-transplantation in hollow (A) and filled (B) conduits (scale bar = 200μm); RT97+ axons (general axon marker) closely associated with transplanted GFP-SCs in the middle of the transplant in hollow (C) and filled (D) conduits (scale bar = 100 μm). CGRP+ axons at the caudal host/SC interface in hollow (E) conduits and in between the layers at the caudal end of filled (F) conduits.

Exp2 (Fig 2): The group without Matrigel (DD) appeared to have the fewest SCs and axons. SC survival appeared to be similar amongst the rest of the groups. More axons appeared to be regenerated into the SC bridges when Matrigel was injected with SCs during transplantation than with pre-coating (DM vs MD). Similar to Exp1, contact guidance of RT97+ and CGRP+ axons was observed among all the groups.

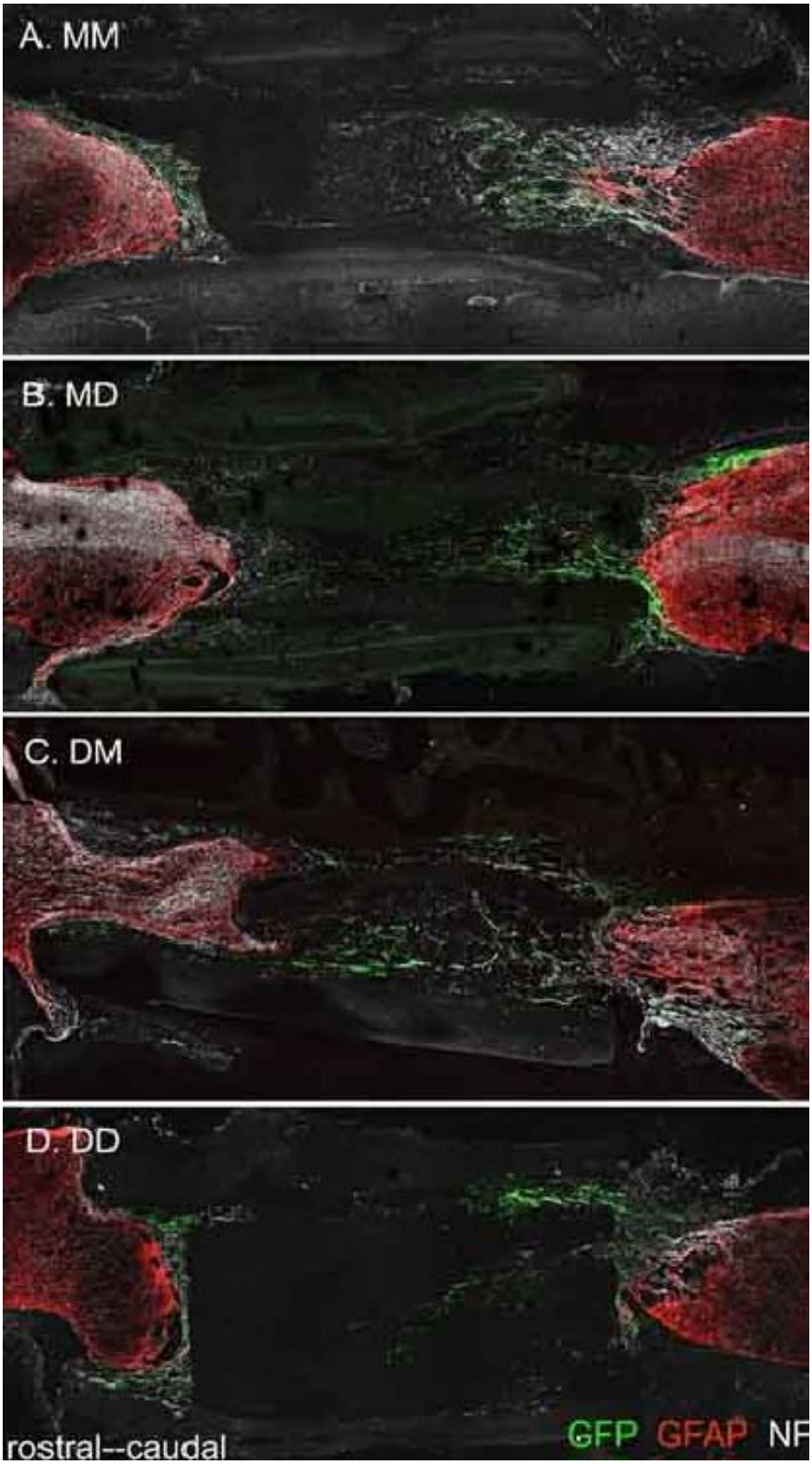

Fig 2. (Exp 2) Confocal fluorescent images of SC bridges 6 weeks post-transplantation in groups MM (A), MD (B), DM (C), and DD (D) (scale bar = 200μm).

Conclusion

This is the first study to investigate filled PVDF-TrFE tubes with SCs for spinal cord repair. The filled fibers enabled contact guidance for directed axon regeneration along the rostral/caudal axis regardless of the use of Matrigel but Matrigel injection enhanced the number of axons entering the fiber filled conduits.

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Investigating Schwann cell growth and neurite extension on PVDF-TrFE scaffolds in vitro

Siliang Wu[1], Yee-Shuan Lee[3], Mary B Bunge[3,4,5], Treena L Arinzeh[2].

[1]Department of Material Science and Engineering, New Jersey Institute of Technology, Newark, NJ, United States; [2]Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, NJ, United States; [3]The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, FL, United States; [4]Departmen of Cell Biology, University of Miami Miller School of Medicine, Miami, FL, United States; [5]Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL, United States

Introduction: Spinal cord injury (SCI) is a devastating condition where effective therapies are needed for recovery of function[1]. Current research has been focusing on various strategies including cell transplantation, electrical stimulation and/or drug or growth factor delivery[2],[3]. Our approach is to combine Schwann cells (SCs) with a piezoelectric scaffold to promote repair. Piezoelectric materials can generate electrical activity in response to minute deformations and SCs secrete neurotrophic factors. Therefore, this combination may stimulate axonal growth. In this study, SC growth and neurite extension on piezoelectric polyvinylidene fluoride-trifluoroethylene (PVDF-TrFE) fibrous scaffolds were evaluated in vitro. In addition, scaffolds were coated with Matrigel, a basement membrane matrix, to evaluate its effect on SC growth and neurite extension.

Methods: 20% PVDF-TrFE (70/30) (Solvay Solexis Inc., Thorofare NJ, US) in methyl ethyl ketone (MEK; Avantor Performance Materials, INC., Center Valley PA, US) was electrospun using the following parameters: 18 gauge needle, 3 ml/h feeding rate, 25 kV power supply, 35 cm distance between collector and tip of needle. Fibers were collected on a collector rotating at 2500 rpm to create an aligned fibrous scaffold. SCs isolated from sciatic nerves of adult female Fischer rats (Harlan Laboratories, Frederick MD, US) were transduced with a lentiviral vector encoding enhanced green fluorescent protein (GFP). SCs were seeded on PVDF-TrFE scaffolds at 15,000 cells/scaffold. Cell viability was evaluated at days 1, 4 and 7 using ATP assay. DRGs were isolated from E17 Sprague-Dawley rat embryos and seeded on to PVDF-TrFE electrospun scaffolds as described previously[4]. DRGs were fixed and stained for neurofilaments at day 2 and viewed using confocal microscopy (Clsi, Nikon, Japan). Both uncoated and Matrigel coated scaffolds were evaluated.

Results and Discussion: SCs seeded on uncoated and Matrigel coated scaffolds were well maintained over time without significant changes in cell number (Fig 1 A, B). Cell attachment was improved by coating the scaffolds with Matrigel, as indicated by significantly higher cell numbers and greater extension of SC processes (Fig 2 A, B). DRGs seeded on Matrigel coated scaffolds showed longer neurite extension (1904.8 ± 228.5 µm) compared to the uncoated group (1361.9 ± 103.5 µm) ($p<0.05$). Uni-directional neurite extension was observed (Fig 2 C, D) on the scaffolds indicating that the orientation of fibers can provide contact guidance cues for promoting neurite outgrowth.

Figure 1A) SC number on uncoated and Matrigel coated PVDF-TrFE scaffolds. Cell number on Matrigel coated scaffolds was significantly higher than on uncoated scaffolds at all time points (*$p<0.05$). B) Average neurite extension of DRGs seeded on uncoated and Matrigel coated PVDF-TrFE scaffolds. Neurite extension on Matrigel coated scaffolds was significantly higher than uncoated scaffolds (*$p<0.05$)

Figure 2 SC-GFP seeded on A) uncoated and B) Matrigel coated PVDF-TrFE scaffolds (60X, scale bar 50 um). DRGs seeded on C) uncoated and D) Matrigel coated PVDF-TrFE scaffolds (RED=neurofilament, 4X, scale bar 500 um.

Conclusion: This study showed that PVDF-TrFE electrospun scaffolds are a promising material for SC growth and neurite extension. Matrigel coated scaffolds showed improved cell attachment and neurite extension which suggests surface modification may be necessary to achieve better outcomes.

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Figure 1

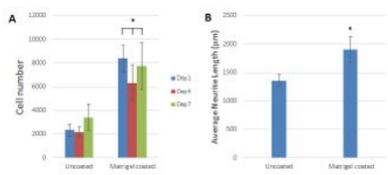

Figure 2