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Combinatorial Tissue Engineering Partially Restores Function after Spinal Cord Injury

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Abstract

Hydrogel scaffolds provide a beneficial microenvironment in transected rat spinal cord. A combinatorial biomaterials based strategy provided a microenvironment that facilitated regeneration while reducing foreign body reaction to the 3-dimensional spinal cord construct. We used poly lactic-co-glycolic acid (PLGA) microspheres to provide sustained release of rapamycin from Schwann cell (SC)-loaded, positively charged oligo-polyethylene glycol fumarate scaffolds. The biological activity and dose-release characteristics of rapamycin from microspheres alone and from microspheres embedded in the scaffold were determined *in vitro*. Three dose formulations of rapamycin were compared to controls in 53 rats. We observed a dose-dependent reduction in the fibrotic reaction to the scaffold and improved functional recovery over 6 weeks. Recovery was replicated in a second cohort of 28 animals that included retranssection injury. Immunohistochemical and stereological analysis demonstrated that blood vessel number, surface area, vessel diameter, basement membrane collagen, and microvessel phenotype within the regenerated tissue was dependent on the presence of SCs and rapamycin. TRITC-dextran injection demonstrated enhanced perfusion into scaffold channels. Rapamycin also increased the number of descending regenerated axons, as assessed by Fast Blue retrograde axonal tracing. These results demonstrate that normalization of the neovasculature was associated with enhanced axonal regeneration and improved function after spinal cord transection.

Keywords

Biodegradable; combination product; nervous system; regeneration; scaffold; spinal cord injury

Introduction

Spinal cord injury (SCI) is a devastating condition that occurs in 250 – 500,000 people each year in the developed world (2013). The poor regenerative capacity of the human central

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

nervous system results from the need to maintain functional stability. This is a biological advantage for a complex nervous system built on billions of interneuronal connections established during growth and development. This contrasts with the simpler peripheral nervous system that effectively regenerates after many types of injury. Failure of repair following SCI results from many factors. Glial and stromal scarring formed at the lesion site block axon growth and there is an increase in inhibitors associated with myelin debris and proteoglycan deposition in the lesion environment (Filbin 2003; Silver and Miller 2004). Acute trauma is followed by a local inflammatory response that causes additional tissue damage. This inflammatory and fibrotic response effectively walls off the injured area and protects surrounding surviving spinal tissue.

The intrinsic capacity for central axons to regenerate was demonstrated by Aguayo and colleagues more than 35 years ago (Richardson *et al.* 1980) We have developed a rationally designed combinatorial system to harness this intrinsic regenerative capacity. A biodegradable hydrogel scaffold and Schwann cells provide key elements of architecture and cellular support for a regenerative environment. Scaffold repair in a spinal cord transection model allows manipulation and study of individual components of the microarchitecture and microenvironment in the regenerating spinal cord. Although it does not mimic acute blunt trauma to the spinal cord, it provides a system where the regeneration process can be deconstructed and the effect of varying individual components of the regeneration environment can be systematically studied.

Using this approach, we previously demonstrated that positively charged oligo[poly(ethylene glycol) fumarate] (OPF +) scaffolds, loaded with or without Schwann cells, facilitated a regeneration permissive lesion environment (Hakim *et al.* 2015) addressing individual barriers, axonal guidance cues, and critical relationships between axon densities and the neovasculature. Barriers to axonal regeneration including the total area of stromal scarring, cyst formation, astrocyte reactivity, chondroitin sulfate proteoglycan (CSPG) deposition and myelin debris were all significantly reduced in rat spinal cords implanted with OPF+ scaffolds compared to animals with transection injury alone. The implanted Schwann cells migrated out of the scaffolds into the adjacent spinal cord and formed into cellular alignments, which we hypothesized may channel regenerating axons at the scaffold borders. This scaffold model supported an association between vascular distribution and axonal regeneration. Using unbiased stereology to provide physiological estimates of blood vessel morphology and distribution, we demonstrated that the formation of smaller, densely packed vessels which resemble the capillary structure in normal spinal cord was significantly associated with increased axonal regeneration (Madigan *et al.* 2014).

We demonstrated significant axonal regeneration but not restoration of function. Over time OPF + scaffold channels became progressively occluded with collagen and fibrotic scarring. This demonstrated a need to reduce the foreign body reaction to implanted scaffolds as a key next step towards improving functional recovery. This foreign body response has been observed with many natural (Gao *et al.* 2013; Gros *et al.* 2010; Horn *et al.* 2007; Jain *et al.* 2006; King *et al.* 2010) and synthetic (Bakshi *et al.* 2004; Gautier *et al.* 1998; Hejcl *et al.* 2009; Tsai *et al.* 2004; Wong *et al.* 2008; Xu *et al.* 1999) biomaterials implanted into the spinal cord. Strategies to reduce scarring, such as methylprednisolone (Chen *et al.* 1996;

Chvatal *et al.* 2008) and chondroitinase ABC to degrade CSPGs (Fouad *et al.* 2005; Hwang *et al.* 2011; Morice *et al.* 2002), have had limited success. Rapamycin is another potential therapeutic candidate, as a potent immunosuppressive drug widely used to prevent allograft organ transplant rejection. Rapamycin has also been used experimentally to reduce fibrosis in reaction to various implanted synthetic materials (Morice *et al.* 2002; Moses *et al.* 2003). The drug has also been shown to reduce microglial activation, astrocyte reactivity, macrophage/neutrophil infiltration and TNF- α secretion in the SCI lesion environment (Goldshmit *et al.* 2015; Tateda *et al.* 2017). It induces autophagy and activates the Wnt/ β -catenin signaling pathway while preserving neurons and promoting motor functional recovery after SCI (Gao *et al.* 2015). Rapamycin may also have a role in normalizing blood vessel distribution as has been proposed in cancer therapy.

We now hypothesize that a novel strategy using sustained, local release of rapamycin directly from implanted OPF + scaffolds would lead to functional neurologic recovery. Our aim is to build upon rational, combinatorial, tissue engineering strategies by delivering rapamycin from microspheres to reduce the foreign body reaction and enhance normal vascularization of regenerating tissue (Guba *et al.* 2002; Jain 2001; Jain 2005). In this study, the parameters for rapamycin release kinetics and cellular responses to dosing are first optimized *in vitro*. An initial cohort of five animal groups then compares the effect of three rapamycin doses compared to controls, using areas of fibrosis and locomotor recovery as outcome measures after complete SCI and scaffold implantation *in vivo* over 6 weeks. A second cohort of three animal groups then carries forward the optimal dosing parameters from the initial animal groups in order to replicate findings of functional recovery and expand a mechanistic study. Detailed stereologic analyses of the developing neovasculature are combined with retrograde axonal tracing to determine the neuronal cell origins of regenerating axons. Locomotor behavioral studies include complete retranssection at 6 weeks.

Materials and Methods

Dose finding studies for rapamycin *in vitro*

We first determined the dose of rapamycin required to inhibit fibroblast proliferation *in vitro* using normal rat kidney (NRK) 49-F fibroblast cells and 0 – 1000nM doses of rapamycin (supplemental methods SM 1 and figure S1). 30,000 fibroblast cells per well were plated in 24 well tissue culture plates were serum-starved by changing the media to DMEM with 0.1% FBS for 24 hours. Fibroblasts were stimulated to proliferate by treatment with 5 ng/mL recombinant human transforming growth factor β 1 (TGF β 1) (R&D Systems, Minneapolis, Minnesota, USA) in DMEM with 0.1% FBS. Control cells were maintained in low serum media only. After two days in culture, fibroblast proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation MTS Assay kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. We then determined whether doses of rapamycin that inhibited fibroblast proliferation were permissive for axonal growth from rat dorsal root ganglion explants *in vitro* by measuring the average longest neurite arc in each explant using NIH ImageJ analysis software for each treatment group at 24 and 48 hours. (supplemental methods SM 2 and figure S1).

Since Schwann cells (SC) were going to be used as supportive cells that would provide extracellular matrix and neurotrophic factors, it was necessary to demonstrate that rapamycin would not disrupt their function. This was assessed using primary SCs isolated from sciatic nerves of postnatal day four rats. SCs were seeded at a density of 30,000 cells per well into 24 well plates with and treated with rapamycin doses ranging from 1 nM to 1 mM or ethanol vehicle in quadruplicate for 3 days at 37°C. SC proliferation was then measured using the CellTiter 96 AQueous One Solution Cell. We measured the production of brain-derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF) production in the supernatant of these SC cultures exposed to the same dose range of rapamycin (supplemental methods SM 3 and figure S1). The purity of the primary SC cultures was determined by immunohistochemistry using anti-rat p75 neurotrophin receptor (p75(NTR) antibodies (supplemental methods SM 4 and supplemental figure S2).

Fabrication, encapsulation efficiency, release kinetics and biological activity of rapamycin in poly-lactic-co-glycolic acid (PLGA) microspheres loaded into positively charged oligo[poly(ethylene glycol)fumarate] OPF+ scaffolds

A microsphere-based drug delivery strategy was used to provide sustained release of effective doses of rapamycin. Poly-lactic-co-glycolic acid (PLGA) microspheres were fabricated using an oil-in-water emulsion-solvent evaporation technique (supplementary methods SM 5.). Encapsulation efficiency of rapamycin within 0.25 mg, 0.5 mg and 1 mg per 250 mg PLGA loaded microspheres was determined by extracting the drug and quantitating its concentration against a standard curve (supplementary methods SM 6 and figure S3). Release kinetics were determined by measuring the amount of rapamycin remaining in microspheres that had been in phosphate buffered saline with 10% fetal bovine serum at 37°C after 1, 2, 3, 4, and 5 weeks (supplementary methods SM 7 and figure S3). Based on these results, rapamycin loaded PLGA microspheres were fabricated in positively charged oligo[poly(ethylene glycol)fumarate] (OPF+) hydrogel scaffolds (supplementary methods SM 8). Microspheres containing different doses of rapamycin were constructed to contain 0.25 mg, 0.5 mg, 1 mg, or 4 mg of rapamycin per 250 mg PLGA. It was important to establish that the biological activity of rapamycin was preserved during the process of microsphere encapsulation, scaffold fabrication and release. The fibroblast proliferation assay described above was used (supplemental methods SM 9 and figure S3).

Scaffold cell loading

OPF+ scaffolds incorporating PLGA microspheres were loaded with 8 µL of Matrigel containing 10⁵ cells/µL Schwann cells (SC) or Matrigel (MG) alone as previously described (Chen *et al.* 2011; Hakim *et al.* 2015). The loaded scaffolds were then incubated in Schwann cell media for 24 hours at 37°C in 5% CO₂ before implantation into animals.

Animal allocation for spinal cord implantation of OPF+ scaffolds embedded with rapamycin-PLGA microspheres

A total of 83 adult female Fischer rats (Harlan Laboratories, Indianapolis, Indiana, USA) weighing approximately 200 g were used in this study, with 53 animals used in the first cohort of animals and 30 in the replication cohort. 75 rats survived the entire length of the experiment, with 6 early animal deaths in the first cohort and 2 early animal deaths in the

second cohort due to complications of surgery or within one week after surgery. All animals had complete spinal cord transection at the T9 level followed by implantation of OPF+ scaffolds. The seven scaffold channels were loaded with matrigel alone or with Schwann cells suspended in matrigel. The walls of the scaffold were loaded with empty PLGA microspheres or microspheres containing three different doses of rapamycin (Table 1). There was a 6 week endpoint in the first experimental cohort. Weekly locomotor scores were assessed and following which scaffolds were embedded and sectioned in the transverse or longitudinal plane at their midpoint for Masson's trichrome staining and immunohistochemical studies.

Thirty additional animals were used in a second cohort with a 6 week endpoint (Table 1). Two animals died during surgery, leaving 28 animals for the cohort. This was designed to replicate the functional outcome findings following transplantation of OPF+ scaffolds with Schwann cell and an optimized dose of rapamycin (1 mg per 250 mg PLGA). These animals were also designated for retrograde fast blue injection studies, spinal cord retranssection, somatosensory evoked potential neurophysiology, and vascular studies.

Animal care, spinal cord transection and scaffold implantation surgeries.

All animal procedures were performed according to the guidelines of the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). Animals were kept on a standard 12 hour light-dark cycle with access to food and water *ad libitum* in conventional housing in accordance with National Institutes of Health (NIH) and U.S. Department of Agriculture guidelines. Animal spinal cord transection surgical techniques, scaffold implantation and post-operative care were as we have previously described (Hakim *et al.* 2015). Animals received perioperative analgesia for 1 week using oral acetaminophen (Mapap, Major Pharmaceutiacals, Livonia, Michigan, USA), oral Baytril (Bayer Corporation, Shawnee, Kansas, USA) and subcutaneous Buprenex (Reckitt Benckiser Pharmaceuticals Inc, Richmond, Virginia, USA). Animals were anesthetized for surgery with intraperitoneal ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and xylazine (Lloyd Laboratories, Shenandoah, Iowa, USA). Animals were randomly assigned to experimental groups and surgeries were performed by a surgeon blinded to animal groups using our previously described approach (Hakim *et al.* 2015; Madigan *et al.* 2014). Laminectomy was performed at the T9-T10 vertebral level and the spinal cord was completely transected using a number 11 scalpel blade. Complete transection was verified with a hooked probe. After transection, the two cut ends of the spinal cord retracted to form a two mm gap. Prepared OPF+ scaffolds were implanted between the free ends of the spinal cord, with the scaffold channels oriented parallel to the length of the spinal cord. After surgery animals were kept in low-walled cages to allow easy access to food and water. Bladders were expressed three times daily after surgery and animals were given antibiotics and analgesics as necessary. All rats were cared for 24/7 by veterinarians and technicians with experience in the management of rat spinal cord injury.

Open field hind limb locomotor function testing

Hind limb function of scaffold-implanted animals was evaluated weekly through open field testing by two to four independent observers blinded to the animal groups and scored using

the 21 point Basso, Beattie, and Bresnahan (BBB) locomotor rating scale (Basso *et al.* 1995). The BBB score for each left and right hind limb was obtained by averaging the scores given by the independent observers. Scores for the left and right hind limbs were then averaged to obtain the overall BBB score for each animal, which was used for statistical analyses. As in previous our previous studies (Chen *et al.* 2017; Hakim *et al.* 2015) any animal with a BBB score greater than 4 (representing slight movement of all three joints of the hind limb) at one week post implantation was considered to have a physiologically incomplete transection and was excluded from further functional analysis. A total of 10 rats distributed across all groups from both cohorts were excluded in this way (Table 1). Spinal cords of the excluded animals in the first cohort were processed and included in subsequent histological analysis.

Tibial SSEP monitoring

Somatosensory evoked potentials (SSEPs) were recorded from animals in the second cohort through subcutaneous scalp needle electrodes following bilateral tibial nerve stimulation. Baseline SSEPs were recorded bilaterally before scaffold implantation surgery, and post implantation at 1, 6, and 7 weeks in 6 retransection animals (supplemental methods SM 10 and figure S5).

Fast Blue retrograde tracer dye injection and spinal cord retransection surgeries.

Ten animals from both groups of the second cohort underwent a second surgery 5 weeks after scaffold implantation for Fast Blue (FB) retrograde tracer analysis (Chen *et al.* 2009; Chen *et al.* 2017). The spinal cord was re-exposed at the level of the prior implantation and 0.6 μ L of Fast Blue dye was stereotactically injected into the spinal cord with a Hamilton syringe through a second laminectomy 5 mm rostral (5 animals/group) or caudal (5 animals/group) to the implanted scaffold. Fast Blue dye was slowly injected over 30 seconds. The needle was left in place for another 30 seconds to prevent dye leakage. The muscle and skin was sutured closed as described for the transection surgery. Animals were allowed to recover for an additional 7 days before tissue harvest and analysis. Since it was important to demonstrate that functional recovery was due to axonal regeneration, six animals in the medium dose group of the second cohort underwent a second surgery at week 6. The spinal cord was re-exposed at the original laminectomy site and the implanted scaffold was visualized. The laminectomy site was slightly expanded rostrally to allow for a retransection of the spinal cord immediately adjacent to the scaffold. Complete transection was verified with a hooked probe. The muscle and skin was sutured closed as described for the original transection surgery.

TRITC-dextran tail vein injections

TRITC-dextran infusion was used to analyze vascular perfusion in the regenerating tissue. A tail vein injection of 250 μ L of a 0.9% saline solution containing 10 mg/mL TRITC-dextran (average molecular weight 65000–85000 Da, Sigma) was performed in the second cohort with Fast Blue dye injections two hours before sacrifice at week 6. Animals were placed in a restraint device and warmed under a heat lamp until the tail vein dilated. The tail was scrubbed with ethanol, one vein was selected and 250 μ L of fluid injected through a 27

gauge needle. The needle was then removed and pressure was applied to the tail using sterile gauze for 30 seconds.

Tissue preparation and sectioning

At the conclusion of the final functional studies, animals were humanely euthanized with an intraperitoneal injection of 0.4 mL sodium pentobarbital (40 mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa, USA). For paraffin tissue embedding and sectioning, spinal cord segments were prepared from animals following transcardial perfusion with 4% paraformaldehyde in PBS as previously described (Hakim *et al.* 2015). For frozen sectioning of Fast Blue dye injected animals with tail vein injections of TRITC-dextran, the vertebral column with spinal cord was removed *en bloc* without animal perfusion and post fixed for 4 days in 4% paraformaldehyde with 10% sucrose at 4°C. The spinal cord was then dissected from the vertebral column and cryoprotected in 30% sucrose at 4°C for 24 hours before being processed for cryostat embedding. The spinal cord length was cut sectioned into 1.5 cm segments designated P1, P2 and P3 moving rostrally, and S1 and S2 moving caudally. The P1 segment contained the OPF+ scaffold. These segments were then embedded with Tissue Freezing Medium (TFM) (Triangle Biomedical Sciences, Durham, North Carolina, USA), cut longitudinally into 30 µm sections (Reichert HistoSTS Cryostat Microtome) and mounted on numbered slides with Aqua-Mount (ThermoFisher).

Analysis of collagen scarring within channels

Slides with transverse tissue sections were selected from the central portion of the scaffolds for staining with the Masson trichrome kit (ThermoFisher). Acquired images were analyzed using the Neurolucida software (MBF Bioscience, Williston, Vermont, USA). A digital grid with 100 µm divisions was overlaid on the tissue, and digital markers for collagen scar or unscarred tissue were placed at each grid intersection within the scaffold channels. The area of collagen scar and unscarred tissue within scaffold channels was then calculated using the Cavalieri probe in the Stereo Investigator software (MBF Bioscience), as previously described (Hakim *et al.* 2015)

Immunohistochemistry

Paraffin transverse sections were selected for immunohistochemistry from the central portion of the scaffolds, adjacent to the Masson trichrome stained slides. Sections were deparaffinized with xylene, rehydrated with graded ethanol and rinsed with distilled water. Heat mediated antigen retrieval was performed by incubating the sections in 1 mM EDTA in PBS for 30 min in a rice steamer. After blocking with 10% normal donkey serum (NDS) in PBS for 30 min sections were incubated overnight at 4°C in primary antibody diluted in 5% NDS with 0.3% Triton X-100 in PBS. Sections were washed with 0.1% Triton X-100 in PBS before incubating for one hour in secondary antibody diluted in 5% NDS with 0.3% Triton X-100 in PBS. After washing with 0.1% Triton X-100 in PBS, sections were mounted with SlowFade Gold Antifade Reagent with DAPI (Molecular Probes, Eugene, Oregon, USA). For DAB (3,3'-diaminobenzidine) staining with nickel enhancement, the DAB substrate kit (Vector Laboratories, Burlingame, California, USA) was used according to the manufacturer's instructions and slides mounted with Aqua-Mount.

Primary antibodies were used against platelet derived growth factor receptor β (PDGFR β) (Rabbit anti-rat, 1:50, Cell Signaling Technology, Danvers, Massachusetts, USA); rat endothelial cell antigen-1 (RECA-1) (Mouse anti-rat, 1:100, Abcam, Cambridge, Massachusetts, USA); p75(NTR) (Rabbit anti-rat, 1:800 Promega) and collagen IV (rabbit anti-rat, 1:300 (Abcam)). Secondary antibodies included Alexafluor 647-conjugated donkey Anti-Rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, Pennsylvania, USA); Cy3-conjugated donkey anti-rabbit IgG antibody (1:200, Millipore, Billerica, Massachusetts, USA); Cy3-conjugated donkey anti-mouse IgG (1:200, Millipore), and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:200, Millipore).

Analysis of Schwann cell number and phenotype *in vivo*

For Schwann cell *in vivo* analysis, transverse sections were selected from the central portion of the scaffolds for staining with p75(NTR) to identify Schwann cells. Automatic object detection was then used in the Neurolucida software (MBF Bioscience, Williston, Vermont, USA) to draw contours around the p75(NTR) stained Schwann cells within the scaffold channels. The sum of the contoured areas outlined within the scaffold channels with automatic object detection was then used for analysis as previously described (Hakim *et al.* 2015).

Microscopy

Images used for Masson Trichrome and immunohistochemical analysis were acquired on a modified Zeiss Axioimager A-1 microscope (Zeiss, Thornwood, New York, USA) equipped with a motorized specimen stage for automated sampling (Ludl Electronics; Hawthorne, New York, USA) and a QICAM 12-bit Color Fast 1394 camera (QImaging, Surrey, British Columbia, Canada). FB- labelled neurons were imaged using C-Apochromat 10 $\times$ and 20 $\times$ objective lenses on a Zeiss LSM510 laser scanning confocal microscope. Additional confocal images were acquired using a LSM 780 confocal microscope (Zeiss, Thornwood, New York, USA).

Sterological analysis of collagen IV stained blood vessels

Surface density (Sv) and length density (Lv) of blood vessels were calculated using unbiased stereology by applying linear and plane grids, respectively, to count vessels as previously described (Madigan *et al.* 2014). Mean vessel diameter (d) was calculated from the ratio of surface to length density, according to the equation $d = Sv/(Lv \times \pi)$.

Quantification of endothelial and perivascular cell coverage of vascular structures

Fiji image analysis software (Schindelin *et al.* 2012) was used to identify the endothelial and perivascular cell coverage of each individual vessel within scaffold channels. A high definition interactive pen display, the Cintiq 24HD touch (Wacom Technology Corporation, Vancouver, Washington, USA) was used by an observer blinded to animal group to draw a contour along the pericyte and endothelial cell staining surrounding the lumen of the vessel. Each contour was labeled and measured in length. After calculating a pericyte to endothelial cell ratio for every individual vessel within a channel, the ratios were divided into 5 clusters according to the extent of pericyte coverage. i.e. <0.75, 0.75–1.5, 1.5–2.25, 2.25–3.0, 3.0–

3.75, 3.75–7.0, or >7.0. After quantifying how many vessels of each cluster were present in each channel, a mean number of vessels per channel were calculated for each animal. Animal groups were compared using a two-way analysis of variance (ANOVA). Posthoc analysis was performed by Newman-Keuls multiple comparison method.

Analysis of vascular function using TRITC-dextran

In the animals with TRITC-dextran tail vein injection, quantification of the amount of intravascular TRITC dye was performed by measuring the surface areas of red fluorescence within longitudinal image fields. Images were systematically taken at 20× magnification spanning the width of the central scaffold channel only of the seven-channel scaffolds to ensure consistency of sampling. The total surface area of intravascular TRITC dye was calculated by setting a defined sensitivity for object detection in the stereology software, which was kept constant across all sections analyzed. Automatic object detection was used for this quantification in the Neurolucida software suite (MBF Bioscience) by manually drawing contours around intense TRITC dye foci within using the same settings of 70.2% sensitivity and highest point density for all animals. The output from object detection software listed the individual surface areas for each fluorescent object, which were summed for each animal and then analyzed as mean area per group.

Quantification of Fast Blue dye labeled regenerated neurons

Fast Blue (FB)-labeled neurons were quantified as previously described (Chen *et al.* 2009; Chen *et al.* 2017) within 30 micron thick longitudinal frozen sections. Labeled neurons in the P1-P3 and S1-S2 (Fig. 6 A, B) segments that were clearly identified as having a visible nucleus and typical cell body morphology were counted within every other section by observers blinded to the animal groups. 158 nuclear diameters were measured in their longest dimension in FB-labeled neurons using NIH ImageJ software in random spinal cord segment images from both animal groups. The mean nuclear diameter (7.43 ± 0.16 microns) was then used in Abercrombie's formula (Abercrombie 1946; Guillery 2002) for FB-neuron count correction, to account for the number of neuronal profiles divided into two by the microtome and thus present in two adjacent sections. The formula provides a ratio of the estimated number of nuclei to the observed number, $T/T+h$, where 'T' is the section thickness and 'h' is the mean nuclear diameter. A calculated correction factor of 0.802 was then derived and applied to the observed counted profile number in each spinal cord segment, indicating that the overestimation of counts was 24.7%. Neuron counts from each 1.5 cm spinal cord segment in the rostral cord (P1+P2+P3) after caudal dye injection and in the caudal cord (S1+S2) after rostral dye injection were then summated to obtain the total number of regenerating neurons. Neurons counted within the P1 segment, which was longitudinally bisected by the OPF+ scaffold, were separated into rostral and caudal counts.

Statistical analysis

All analyses were performed by observers blinded to treatment groups. The averages for proliferation of fibroblasts and Schwann cells, Schwann cell BDNF and GDNF production, DRG neurite outgrowth, percent channel area without collagen scarring, channel blood vessel length, channel blood vessel surface area, channel blood vessel mean diameter, and hind limb function in the subset of animals that received retranssections were all analyzed by

one-way ANOVA with *post hoc* analysis using Newman-Keuls multiple comparison test. The average BBB scores for hind limb function in the first and second cohorts and pericyte to endothelial cell ratios of scaffold blood vessels were analyzed by two-way repeated measures ANOVA with *post hoc* analysis using Newman-Keuls multiple comparison test. Rostral and caudal total Fast Blue labeled neuron counts and areas of TRITC dye foci were analyzed using the nonparametric Mann-Whitney test. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, California, USA). All data are presented as mean $\pm$ standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

Results

Rapamycin inhibits fibroblast and Schwann cell proliferation and stimulates neurotrophin production

Rapamycin inhibited fibroblast proliferation between 1 nM and 100 nM without affecting cell viability (Supplementary Fig. 1 A). Neurite outgrowth from dorsal root ganglion explants in 1 nM to 100 nM rapamycin was unaffected by drug treatment after 24 and 48 hours (Supplementary Fig. 1 B and C). Doses of 1 nM to 1 mM rapamycin inhibited proliferation of primary rat SC *in vitro* over 3 days (Supplementary Fig. S1 D). BDNF concentration in Schwann cell conditioned media (pg/ml, normalized to MTS absorbance values) increased with rapamycin doses ranging from 1 to 1000 nM over vehicle control Schwann cells after 72 hours of drug treatment. GDNF concentration increased only after treatment with 1000 nM rapamycin (Supplementary Fig. 1 E and F). Primary SC were used after four passages to simulate cells loaded into hydrogel scaffolds for *in vivo* studies. 90.4 $\pm$ 0.5% of these SCs maintained p75(NTR) expression (Supplementary Fig. S2). Morphological changes of Schwann cells were not observed over 3 days in culture in the presence of rapamycin.

Rapamycin released from PLGA microspheres and OPF+ scaffolds inhibits fibroblast proliferation

Based on the results above, PLGA microspheres containing 0.25 mg, 0.5 mg, 1 mg or 4 mg of rapamycin per 250 mg PLGA were fabricated. The encapsulation efficiency of five mg of microspheres containing 0.25 mg, 1 mg, and 4 mg of rapamycin per 250 mg PLGA was $104 \pm 9\%$, $100 \pm 10\%$, and $107 \pm 8\%$, respectively; kinetics of drug release from ten mg of each of these three microsphere doses were determined over 5 weeks (Supplementary Fig. S3 A). Five micrograms of the 0.25 mg, 0.5 mg and 1 mg rapamycin per 250 mg PLGA microspheres inhibited NRK fibroblast proliferation over 4 weeks (Supplemental Fig. 3 B). TGF- β 1-stimulated fibroblast proliferation was prevented *in vitro* when the same three formulations of rapamycin-PLGA microspheres were incorporated into the oligo[poly(ethylene glycol) fumarate] (OPF+) hydrogel scaffolds at a density of 1 mg microspheres per 10 μ l liquid OPF+ in 2 mm length multichannel scaffolds (Supplementary Fig. 3 C). OPF+ scaffolds embedded with 0.25 mg, 1 mg and 4 mg rapamycin per 250 mg PLGA were used in the animal studies.

Rapamycin from PLGA microspheres in OPF+ scaffolds reduces fibrosis after SCI

OPF+ scaffolds were implanted into five groups of Fischer rats after spinal cord transection at the T9 level (Table 1). The first cohort of 53 animals was used for a rapamycin dose response study with scaffolds containing primary SC compared to SC-loaded scaffolds with empty microspheres and scaffolds with no cells and empty microspheres. All animals were sacrificed at 6 weeks post implantation for tissue sectioning and analysis (Table 1).

At 6 weeks scaffolds containing empty PLGA microspheres, with or without SC developed dense collagen scarring throughout channels (Fig. 1 A and B). Low dose rapamycin animals were similar (Fig. 1 C). Both medium and high dose rapamycin had significantly less channel collagen deposition (Fig. 1 D and E). $11.54 \pm 0.91\%$ and $10.77 \pm 1.38\%$ of the core channel area remained clear of collagen in the Matrigel-only (n=8) and SC-loaded scaffolds with empty microspheres (n=8) groups, with $8.01 \pm 2.40\%$, $50.35 \pm 8.45\%$, and $62.04 \pm 2.85\%$ of the channel area clear in the SC-loaded scaffolds with low dose (n=4), medium dose (n=9) and high dose rapamycin (n=7) groups, respectively (Fig. 1 F).

Rapamycin influences Schwann cell number and phenotype *in vivo*

SCs were identified by p75(NTR) staining in transverse sections through the central portion of the scaffolds (Supplementary Fig. 4 A–E). There was an increased area of p75(NTR) staining in animals implanted with SC-loaded scaffolds with empty microspheres compared to other groups (Supplementary Fig. 4 F). SCs in rapamycin-treated channels exhibited a more differentiated, spindle shaped morphology compared to the large amoeboid morphology seen in channels without rapamycin.

Rapamycin promotes functional recovery in two separate animal cohorts

We next determined the effect of rapamycin treatment on functional locomotor recovery in the first cohort (Table 1). After 4 weeks, rats implanted with SC-loaded scaffolds containing medium dose rapamycin microspheres (SC+Medium RAPA-MS) demonstrated improved functional recovery (BBB score 4.69 ± 0.57 , n=9 animals) when compared to animals implanted with Matrigel-only scaffolds with empty microspheres (1.58 ± 0.57 , n=6) ($p=0.0059$) (MG+Empty-MS; Fig. 2 A). After 5 weeks, the mean score in the medium dose rapamycin group was 5.89 ± 0.45 , further improved over the MG+Empty-MS group (1.83 ± 0.52) ($p=0.0001$) and the SC-loaded group with empty microspheres (SC+Empty-MS; 2.75 ± 0.75) ($p=0.0012$). At week 6, the mean score in SC+Medium RAPA-MS was 5.72 ± 0.53 , compared with MG+Empty-MS (1.17 ± 0.25 ; $p<0.0001$), and SC+Empty-MS (3.08 ± 0.86 ; $p=0.0016$). To replicate these results a second cohort of 28 animals (Table 1) was used; SC+Medium RAPA-MS (1 mg rapamycin per 250 mg PLGA; n=16 animals), and MG+Empty-MS (n=12 animals). Blinded weekly measurement of BBB locomotor function demonstrated divergence of functional recovery at the 4-week time point (Fig. 2 B). The mean BBB score in the SC+Medium RAPA-MS group (3.16 ± 0.52) (n=15) was higher than that measured in the MG+Empty-MS (1.06 ± 0.25) (n=11) ($p<0.01$). At week 6 the mean score in the SC+Medium RAPA-MS group was 4.91 ± 0.46 compared to the MG+Empty-MS group (3.23 ± 0.58) ($p<0.05$).

To confirm that functional improvement was related to regeneration through the implanted scaffold, 6 animals from the SC+Medium RAPA-MS group underwent spinal cord retranssection at the rostral tissue-scaffold interface at 6 weeks post implantation. One week later BBB scores were 1.72 ± 0.36 compared to 4.64 ± 0.95 ($p = 0.011$) at week 6 (Fig. 2 C). Histological assessment of the retranssection site confirmed that compared to week 6 animals without retranssection (Fig. 2 D), week 7 animals showed tissue disruption at the rostral tissue-scaffold interface with a characteristic fibrinoid scar (arrow) resembling that observed at week 1 post transection injury as previously reported (Hakim *et al.* 2015) (Fig. 2 E).

Somatosensory evoked potential measurement

Pre-operative waveforms were present in all animals at baseline. SSEP waveforms were scored to be absent or likely absent (mean scores of 2.045 ± 0.142 in MG+Empty-MS and 1.87 ± 0.11 in SC+Medium RAPA-MS) at 1 week post implantation, confirming the complete nature of the transection injury. Definitive restoration of SSEP waveforms was not observed in either group at 6 weeks post implantation, and no change in the scoring was seen between week 6 and week 7 retranssection measurements. (Supplementary Fig. 5).

Rapamycin and Schwann cells influence vascularization, vessel architecture and function

Vascularity of scaffold channels was assessed by collagen IV staining of blood vessel basement membranes in transverse sections through the central portion of the scaffolds in the first cohort of animals (Fig. 3 A–E). Blood vessel length (a sum function of the number of blood vessels), mean vessel surface area, and mean diameter were analyzed through unbiased stereology (Fig. 3 F–H). SC+Empty-MS had a higher vessel length ($482.5 \pm 57.09 \mu\text{m}$) and vessel surface area ($12,034 \pm 775.6 \mu\text{m}^2$) than MG+Empty-MS ($192.2 \pm 27.61 \mu\text{m}$ and $3,341 \pm 516.3 \mu\text{m}^2$, respectively) ($p < 0.05$ for each comparison). Blood vessel length in the SC+Low RAPA-MS ($626.6 \pm 104.9 \mu\text{m}$) and SC+Medium RAPA-MS ($475.3 \pm 103.8 \mu\text{m}$) groups were statistically equivalent to the SC+Empty-MS group. Vessel surface area was similarly equivalent in the SC+Low RAPA-MS ($12,983 \pm 2,397 \mu\text{m}^2$) and SC+Medium RAPA-MS ($9399 \pm 2203 \mu\text{m}^2$) groups compared to the SC+Empty-MS group. The presence of Schwann cells with no rapamycin or with low and medium rapamycin positively influenced blood vessel formation in OPF+ channels. However, vessel length ($168.0 \pm 53.96 \mu\text{m}^2$) and surface area ($3,762 \pm 1,379 \mu\text{m}^2$) in SC-loaded scaffolds eluting the high dose of rapamycin was equivalent to Matrigel-only scaffolds without rapamycin.

Blood vessel morphology also differed in different conditions. Vessels within SC+Empty-MS scaffolds had an irregular shape and larger mean diameter in contrast to smaller diameter vessels observed in animals with Matrigel-only scaffolds and the rapamycin treated groups (Fig. 3 A–E). The mean blood vessel diameter in SC+Empty-MS was $8.413 \pm 0.76 \mu\text{m}$, in Matrigel-only scaffolds $5.728 \pm 0.84 \mu\text{m}$ ($p < 0.05$), and in SC+Medium RAPA-MS was $6.10 \pm 0.36 \mu\text{m}$ ($p < 0.05$) (Fig. 3 H). Blood vessel diameters in the low and high dose rapamycin groups were also smaller than SC-loaded scaffolds with empty microspheres. Cellular structure of vessel walls varied between conditions. Small diameter vessels with normal endothelial cell staining (RECA-1) and pericyte (PDGFR β) coverage were consistently observed in the channels of animals implanted with SC+Medium RAPA-MS scaffolds (Fig. 4 A). Irregularly bordered vessels with hypertrophic pericytes (Fig. 4 B) and

vessels composed only of pericytes without endothelial cells (Fig. 4 C) were observed in SC +Empty-MS animals consistent with incomplete or irregular vessel formation.

Pericyte to endothelial cell ratio (PC/EC ratio) was calculated for each vessel in a channel. Animals in the SC+Empty-MS group had the highest number of vessels with PC/EC >7.0, (Fig. 4 D). This immature vascular component was found to decrease with increasing RAPA concentration, and was also significantly lower in the MG+Empty-MS group. Animals in the SC+Low RAPA-MS group had the highest number of vessels with a PC/EC ratio of 0.75 to 1.5, representing a normal PC/EC ratio for healthy CNS vasculature (Fig. 4 D).

Vessel function was assessed in the second cohort of the study by tail vein injection of TRITC-dextran (Table 1). Foci of TRITC dye were rarely observed in the central channels of animals implanted with MG+Empty-MS (Fig. 5A). In contrast, the channels of animals implanted with SC+Medium RAPA-MS demonstrated many foci of concentrated TRITC dye as well as areas of more diffuse TRITC expression (Fig. 5B). Surface area measurements demonstrated a more than four-fold increase in TRITC dye signal in the channels of rapamycin treated animals ($113.0 \pm 32.04 \mu\text{m}^2$) compared with the Matrigel-only with empty microspheres group ($26.34 \pm 6.27 \mu\text{m}^2$) demonstrating improved vascular connectivity to the systemic circulation.

Rapamycin treatment results in increased axonal regeneration across implanted scaffolds

One week after FB injection in animals in cohort 2, FB-labeled neuron nuclear profiles were counted within 15 mm spinal cord segments designated P1 – P3 rostral and S1 – S2 caudal (Fig. 6A, B). Cells with morphology resembling large motor neurons and smaller spinal interneurons were identified (Fig. 6 C, D). The Abercrombie formula correction factor was calculated as 0.802. The mean total of neurons rostral to the scaffold (P3 + P2 + rostral P1 segments) after caudal FB injection was higher in SC+Medium RAPA-MS group ($36,326 \pm 15,176$ neurons, n=5 animals) compared to MG+Empty-MS animals ($9,551 \pm 1857$ neurons, n=5 animals) ($p=0.032$) (Fig. 6E). The mean number of neurons counted in the caudal cord (caudal P1 + S1 + S2) after rostral dye injection was $5,602 \pm 676$ in the MG +Empty-MS group (n=5 animals), and $10,592 \pm 2780$ in the SC+Medium RAPA-MS group (n=4 animals) ($p=0.413$) (Fig. 6 F). The majority of counted neurons were in the rostral P3 and P2 segments in animals implanted SC+Medium RAPA-MS scaffolds, whereas the neuron counts in animals without implanted cells or rapamycin were more evenly distributed (Fig. 5G).

Discussion

Combinatorial approaches to restore function after spinal cord injury have been proposed for a number of years (Olson 2013). Systematic testing of individual components to predict and optimize their contribution to a final advanced or combination medical product is a goal of tissue engineering. In this study two different biomaterials (PLGA and OPF+), rapamycin and Schwann cells were combined. We have previously demonstrated that OPF+ supports the most robust axonal regeneration (Chen *et al.* 2011) in our scaffold system. Schwann cells produce the extracellular microenvironment that supports regeneration of CNS axons and promote regeneration in spinal cord grafts (Chen *et al.* 1996) and in OPF+ scaffolds (Chen *et*

al. 2017; Olson *et al.* 2009). Fibrosis and a foreign body response to SC-loaded OPF+ scaffolds were identified as a major remaining barrier to axon regeneration and functional recovery (Hakim *et al.* 2015). Rapamycin has been used to reduce fibrosis in reaction to various implanted synthetic materials (Morice *et al.* 2002; Moses *et al.* 2003). Combinatorial strategies to reduce scarring have included methylprednisolone (Chen *et al.* 1996; Chvatal *et al.* 2008) and chondroitinase ABC to degrade CSPGs (Fouad *et al.* 2005; Hwang *et al.* 2011; Morice *et al.* 2002). Biomaterials that incorporate nanotopographies including pores and nanofibers, the elution of bioactive molecules from nanoparticles, and viral gene therapies have also been broadly employed as strategies to reduced fibrosis and gliosis, and to alter the profile of recruited macrophages or adaptive T-cell responses (Dumont *et al.* 2016) following SCI. Combination strategies that influence the inter-relationship between the immune response and the developing vasculature has recently been expertly reviewed (Haggerty AE *et al.* 2018). A current focus involves the use of natural extracellular matrices, including fibrin, hyalonic acid, collagen or decellularized matrix, to influence macrophage phenotypes, while simultaneously combining cellular and/or polymer-based delivery of angiogenic factors.

We now demonstrate that combining SC with OPF+ scaffolds that contain PLGA microspheres providing sustained release of rapamycin restores function. Neurologic function improved from complete hind limb paresis immediately after transection, to isolated hip, knee, and ankle movements and limb sweeps over the course of 6 weeks following scaffold placement, according to BBB locomotor functional scale scoring. Our study included a complete replication of these findings in a second cohort of animals. The replication study included a demonstration of abolishing the improvement gains with retransection at 6 weeks, supporting that the improvements were attributable to tissue regeneration. Animals were unable to recover intervals of uncoordinated stepping (BBB scores from 8–13) or forelimb and hindlimb coordination and rearing behaviors (scores 14–21) (Basso *et al.* 1995). Recovery of SSEPs was not observed despite locomotor improvements. We have previously shown (Cloud *et al.* 2012) that the likelihood an evoked potential can be recorded after injury depends upon the area of spared dorsal funiculi tissue at the spinal cord lesion site. BBB scores, in turn, directly correlated to the area of intact tissue, where the lowest BBB scores of 11–12 correlated to approximately 20% normal tissue remaining after a spinal cord hemisection. Failure to recover the SSEP in completely transected animals therefore implies that the dorsal columns did not regenerate or reconstitute to a threshold tissue area in our animal groups.

Differences in SC morphology *in vivo* and neurotrophic factor production *in vitro* were observed with rapamycin treatment. Rapamycin has been previously shown to increase SC migration and nerve growth factor (NGF) secretion *in vitro* (Liu *et al.* 2014) In the current study, rapamycin treatment was also observed to increase BDNF and GDNF secretion by SC *in vitro*. The increases in BDNF and GDNF secretion by transplanted SC could contribute to the observed improvements in axon regeneration and functional recovery with rapamycin delivery from OPF+ scaffold *in vivo*. Further, the observed differences in SC morphology suggest that rapamycin may influence maturation of SC within the lesion environment. A recent study demonstrated that reduction in volume of SC cytoplasm that accompanies myelin maturation is regulated through autophagy and could be influence by rapamycin

(Jang *et al.* 2015). The smaller, spindle shaped morphology of p75(NTR)-positive SC in rapamycin-containing scaffolds may suggest a more mature myelinating phenotype when compared to the larger, amoeboid morphology observed in scaffolds without rapamycin delivery.

Our study was limited to some extent by omitting a Matrigel+Rapamycin microspheres only control group. Our primary study focus was to determine the combinatorial or additive effects between the drug and implanted cells, rather than between the drug in empty scaffolds. We have consistently shown that Matrigel only scaffolds are capable of supporting a rate of axonal regeneration that is approximately 80% less than that seen with SC implanted scaffolds without drug elution (Madigan *et al.* 2014, Hakim *et al.* 2015, Chen *et al.* 2018). The effect of rapamycin on astrocytes, including on astrocyte infiltration into the scaffold and on astroglial scar formation, was also not assessed in the current study, and represents another important limitation in the clarification of mechanisms behind the observed functional improvement. We have previously observed (Hakim *et al.* 2015) that reactive astrocytes producing CSPGs form perimeter boundaries around OPF scaffolds with and without SCs in the adjacent spinal cord, but do not infiltrate into scaffold channels. Studies investigating upstream regulators of mTOR activity in knockout animal models have demonstrated role for mTORC1 signaling in astrocyte reactivity and glial scar formation (Fraser *et al.* 2004, Uhlmann *et al.* 2004). Evidence for the mTOR dependence of reactive astrogliosis following SCI was also described in a subsequent study in rat spinal cord astrocytes (Codeluppi *et al.* 2009). Here, increased proliferation and migration of cultured spinal astrocytes in response to growth factor stimulation *in vitro* could be prevented with rapamycin, and systemic rapamycin treatment was able to reduce astrocyte reactivity and migration of GFAP-positive astrocytes into the injury epicenter following ischemic SCI *in vivo*. Jahan *et al.* demonstrated that TGF β treatment of rat cortical astrocytes resulted in hypertrophy and the production of the CSPGs neurocan, brevican, and aggrecan *in vitro* (Jahan and Hannila 2015), and that CSPG production in response to TGF β was inhibited by rapamycin. Thus, rapamycin treatment may also be effective in preventing CSPG production by hypertrophic, reactive astrocytes through inhibition of TGF β and mTor signaling.

The vascular pattern within OPF+ scaffold channels was found to be dependent on whether scaffolds were loaded with SC and the dose of rapamycin delivered through PLGA microspheres. The observation that few vessels were present in the channels of MG-only scaffolds suggests that angiogenic factors produced by SC are crucial for vascularization. Blood vessel number (vessel length) and surface area coverage improved with SC implantation alone and with low and medium dose rapamycin. High dose rapamycin reduced blood vessel number and surface area back down to the levels seen without implanted cells or drug. The effects of high dose rapamycin on SC, pericytes, fibroblasts, or epithelial cells and their interaction or signaling to promote vessel formation in our model was not directly studied here. That high dose rapamycin impaired vascular formation or is anti-angiogenic, by an excessive anti-proliferative effect, autophagy, or suppression of VEGF production, for example, is plausible, and thus the highest dose was deemed suboptimal as part of the dose-response design to carry forward into subsequent studies. The neurotrophic factor BDNF, which is produced by SC *in vitro*, is known to be a potent inducer of angiogenesis (Kermani and Hempstead 2007). Of note, BDNF administration was able to stimulate *in vivo*

neovascularization within Matrigel plugs transplanted subcutaneously in mice (Kermani *et al.* 2005). The neovascularization was found to be mediated through local tropomyosin receptor kinase B (TrkB) activation by BDNF on endothelial cells as well as the recruitment of pro-angiogenic circulating hematopoietic cells. Rat brain derived endothelial cells were observed to express receptors for neurotrophic factors BDNF, NGF, TrkB and p75(NTR), respectively. BDNF activation of TrkB was found to increase endothelial cell survival, tube formation in three-dimensional collagen matrices, and vascular endothelial growth factor (VEGF) receptor expression through activation of PI3K/Akt dependent signaling pathways. In contrast, propeptide NGF (pro-NGF) activation of p75(NTR) signaling before cleavage by metalloproteinases was shown to increase endothelial cell apoptosis. In combination with these previously described effects of BDNF on endothelial cell function, the observation of increased SC within SC-loaded scaffolds at 6 weeks post implantation compared to MG-only scaffolds implicate BDNF as an important potential mediator of neovascularization within OPF+ scaffolds following SCI.

Rapamycin may have a direct effect on endothelial cells participating in graft angiogenesis. Previous studies in malignant tumors have revealed the importance of a balance between local pro- and anti-angiogenic factors in the development of functional vasculature. Anti-angiogenic therapies, such as administration of anti-VEGF antibodies, have been shown to result in smaller diameter, less permeable, and less tortuous tumor vessels with increased perfusion and oxygen delivery in mice bearing vascularized tumor xenografts or allografts (Hansen-Algenstaedt *et al.* 2000; Yuan *et al.* 1996). A hypothesis of vascular normalization proposes that the overabundance of pro-angiogenic factors such as VEGF, TGF β , and PDGF within the tumor microenvironment produces an imbalance of pro- and anti-angiogenic signals leading to the development of abnormal microvasculature (Jain 2001; Jain 2005). More mature and functional vascular phenotypes are restored by exogenous anti-angiogenic treatments. Rapamycin has been shown to have antiangiogenic properties through reducing VEGF secretion by tumors and preventing VEGFR signaling in endothelial cells (Guba *et al.* 2002). It is possible that a process of vascular normalization akin to that described in the cancer literature has occurred with rapamycin treatment in the current study.

This possible explanation of the improvements in vascularization observed with rapamycin treatment is supported by the similarities in perivascular cell phenotypes and vessel morphologies between tumor vasculature and that seen in channels of SC-loaded scaffolds with empty microspheres. Hyperplasia of pericytes is a defining characteristic of the microvasculature in high grade malignant gliomas (Sun *et al.* 2014). Vessel-like structures consisting only of cells with pericyte markers have also been observed in glioma tissue. SC-loaded scaffolds with empty microspheres exhibited similar findings suggesting that alternative mechanisms of pericyte-mediated vessel extension may be involved in the unregulated angiogenesis observed in both tumor and tissue engineering contexts.

Large diameter, irregularly shaped, and hyperpermeable vessels with abnormal pericyte coverage been produced in non-tumor tissue in transgenic mice with inducible expression of a myristoylated form of Akt1 (myrAkt1) (Phung *et al.* 2006). These large and irregular vessels were similar in appearance to those observed in SC-loaded OPF+ scaffolds with empty microspheres. The abnormal vessel phenotype formed over a period of 6–7 weeks

upon myrAkt1 induction and reversed after myrAkt1 suppression for 4 weeks. Concurrent rapamycin treatment almost entirely prevented the development of abnormal vasculature following myrAkt1 induction. These results, may explain the similar effects in vascular normalization observed within OPF+ scaffolds when rapamycin is delivered from PLGA microspheres. Future studies evaluating Akt activity within the scaffold channels and mTOR dependent pathways will be important (Sarbasov *et al.* 2006) to further improve vascularization of biomaterial implants.

In conclusion, this study demonstrates the potential for tissue engineering to bring multiple regenerative strategies to restore function after spinal cord injury. This approach also allows multiple components of the system to be modulated in a controlled and independent way. This includes the site and rate of drug delivery. Microsphere composition can be manipulated to produce timed release at different periods and for different intervals. In the future different types of cells or cells releasing different growth factors in different regions of the scaffolds may be used to regionally regulate regeneration of specific classes of axons. Rational and mechanism-based approaches are available to systematically modulate these processes using combinatorial tissue engineering strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Inhibition of collagen scarring within scaffold channels by rapamycin *in vivo*.

(A) Masson trichrome staining from the midpoint of OPF+ scaffolds demonstrated high levels of collagen scarring (blue) in the channels of animals implanted with MG-only scaffolds with empty microspheres. (B) SC-loaded scaffolds with empty microspheres. (C) SC-loaded scaffolds with low dose rapamycin microspheres. (D) In contrast, a significant amount of unscarred tissue (pink) was observed within the channel centers of animals implanted with SC-loaded scaffolds with medium dose rapamycin microspheres (E) SC-loaded scaffolds with high dose rapamycin microspheres. (F) Percent channel area without collagen scarring in each of these groups was quantitated. (**** $p < 0.0001$) denotes significant decrease from both the SC-loaded scaffolds with medium dose rapamycin microspheres and SC-loaded scaffolds with high dose rapamycin microspheres groups. 500 μm scale bar in panel (A) applies to panels (B) through (E).

Figure 2. BBB scores in two animal cohorts, including spinal cord retransection.

(A) Hind limb recovery over time in open field testing using the BBB scale in cohort 1. (B) Second cohort of scaffold-implanted animals and with SC and medium dose rapamycin. (C) Scaffold implanted animals with retransection at 6 weeks post implantation. Panel A ( ) denotes significant difference from MG-only scaffolds with empty microspheres group while (*) denotes significant difference from both MG-only scaffolds with empty microspheres ($p=0.0001$ week 5, $p<0.0001$ week 6) and SC-loaded scaffolds with empty microspheres groups ($p=0.0012$ week 5 and $p=0.0016$ week 6). At week 6, a significant difference was measured between the SC scaffolds with medium and high rapamycin doses ($p=0.029$). In panel B, (*) denotes significant difference from MG-only scaffolds with empty microspheres group ($p<0.01$ at week 4 and $p<0.05$ at week 6). (D) Longitudinal section through the center of implanted SC-loaded OPF+ scaffolds with medium dose rapamycin microspheres stained with Masson trichrome at 6 weeks post implantation. (E) Similar longitudinal section from an animal 7 weeks post implantation following retransection one week earlier. Longitudinal section (rostral end upward) confirms disruption of the scaffold tissue and channel architecture. Scale bar 500 μ m (A and B).

Figure 3. Vascularity and vessel morphology within scaffold channels *in vivo*.

Images of individual scaffold channels stained for blood vessel basement membrane with collagen IV. (A) Few vessels in MG-only scaffolds with empty microspheres. (B) Large irregular vessels in SC-loaded scaffolds with empty microspheres. (C, D) Many smaller diameter vessels in SC-loaded scaffolds with low dose and medium dose rapamycin microspheres. (E) Few vessels in SC-loaded scaffolds with high dose rapamycin microspheres. 200 μm scale bar (A-E). (F) Quantitative stereological measurements of vessel length. (G) Stereological measurement of vessel surface area. (H) Quantitative stereological measurements of mean vessel diameter. In panels (F) and (G), (*) denotes significant

difference from both MG-only scaffolds with empty microspheres and SC-loaded scaffolds with high dose rapamycin microspheres groups ($p<0.05$). In panel (H), $*p<0.05$ is denoted.

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Fast Blue TRITC-dextran

Figure 4. Vascular phenotype and function within scaffold channels *in vivo*.

Representative high power confocal images of blood vessels stained for endothelial cells with RECA-1 (red) and pericytes with PDGFR β (green). (A) Regular smaller diameter vessels with endothelial cells covered by pericytes characteristic of mature microvasculature in the channels of animals implanted with SC-loaded scaffolds with medium dose rapamycin microspheres. (B, C) Large irregular vessels with hypertrophic pericytes and vessel structures composed only of pericytes without endothelial cells were observed within SC-loaded scaffolds with empty microspheres. (A-C) 20 μ m scale bar. (D) Ratio of pericyte/endothelial cell coverage of capillary lumina estimated by quantitative stereological measurement.

Fig. 5. Representative confocal images of the innermost central scaffold channel in longitudinal sections.

(A) Few foci of TRITC-dextran dye and Fast Blue labeled axons in Matrigel-only scaffolds with empty microspheres group. (B) Multiple TRITC-dextran dye foci in punctate, intravascular (arrow heads) and diffuse leakage patterns were observed in SC-loaded scaffolds with medium dose rapamycin microspheres along with longitudinally-oriented axons labeled with Fast Blue. 50 μm scale bar in panel (A) applies to panel (B). (C) Vascular perfusion quantitated using surface area measurements of intense TRITC dye (** $p<0.01$).

Fig. 6. Retrograde tracing of regenerated axons with Fast Blue dye.

(A) Gross image of the spinal cord of a scaffold-implanted animal with rostral injection of Fast blue dye. Tissue is oriented with rostral up and caudal down, with a metric ruler laid beside for scale. The site of Fast Blue injection can be seen as the tan spot (arrow head) rostral to the translucent OPF+ scaffold (arrow). (B) A schematic demonstrating the labeling of spinal cord segments. (C) Representative images of Fast Blue labeled neurons with characteristics predominately of large motor neurons and (D) characteristics predominately of small spinal interneurons. 50 μ m scale bar in panel (C) applies to panel (D). (E) Quantification of total regenerated neurons labeled with Fast Blue in the combined rostral P1, P2 and P3 spinal cord segments (* $p < 0.05$). (F) Combined caudal P1, S1, and S2 spinal cord segments following caudal and rostral Fast Blue injections, respectively. (G) Quantification of regenerated Fast Blue labeled neurons within each individually counted spinal cord segment (G).

Table 1.**Overview of Animal Study Design**