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Axonal Regeneration Supported by Oligo[poly(ethylene glycol)fumarate] Cell-Loaded Hydrogel Scaffolds in the Transected Rat Spinal Cord

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September 2011
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Abstract

Spinal cord injury results in complete tissue destruction and irreversible loss of neurologic function below the level of the lesion in 40% of patients in Ireland. Tissue engineering using polymer scaffolds offers potential to rebuild neural tissue through the injury site and to re-establish functional connections. An introductory review highlights current tissue engineering strategies and novel therapeutic approaches to axonal regeneration. Given the patient morbidity associated with respiratory compromise, the discrete tracts in the spinal cord conveying innervation for breathing represent an important and achievable therapeutic target. A variety of naturally derived and synthetic biomaterial polymers have been developed for placement in the injured spinal cord. Axonal growth is seen to be supported by inherent properties of the selected polymer, the architecture of the scaffold, permissive microstructures such as pores, grooves or polymer fibres, and surface modifications to provide improved adherence and growth directionality. Structural support of axonal regeneration is combined with integrated polymeric and cellular delivery systems for therapeutic drugs and for neurotrophic molecules.

This thesis proposes that cell-seeded hydrogel polymer scaffolds in a thoracic cord transection model allow for separate and controlled manipulation of the architecture, surface properties, and the molecular and cellular microenvironment of the regenerating spinal cord. The ability to control these variables with precision may enable the scaffold implant to be informative about individual facets of the repair process. A novel hydrogel, oligo[poly(ethylene glycol)fumarate] (OPF) has been developed, integrating chemical modification for positive surface charge as a substrate for axon growth. OPF scaffolds are loaded with either Schwann cells or mesenchymal stem cells, derived from the bone marrow of transgenic rats with expression of the enhanced green fluorescent protein (eGFP-MSCs). The capacity of each cell type to influence the regenerating environment is compared. Control scaffolds contain extracellular matrix only.

Chapter 2 describes the isolation of rat eGFP-MSCs and their characterization as stems cells capable of phenotypic differentiation to mesenchymal lineages. The isolation and characterization of Schwann cells from
neonatal rat pups is also described. OPF polymer synthesis, scaffold fabrication, scaffold cell loading with eGFP-MSCs and Schwann cells, thoracic spinal cord transection surgery and scaffold implantation in rats and postoperative outcomes are shown. Gross pathology of spinal cord specimens demonstrates scaffold integration and alignment.

In Chapter 3, the architecture of tissue formed after 4 weeks in response to the implantation of each scaffold type is examined initially by means of a general histopathology overview. Detailed immunohistochemistry and stereology approaches are then applied to the model. An analysis of the cell types that are contributing to separate structural and functional compartments within scaffold channels is done using antibodies to glial fibrillary acid protein (GFAP), S-100, vimentin, and neuroglycan-2. Image analysis quantifies the proportional area occupied by each cell type. Established astrocitosis is seen in a peripheral channel compartment, involved in producing boundaries which may organize axon growth. A structurally separate channel core contains immature astrocytes, Schwann cells, eGFP-MSCs, blood vessels and regenerating axons. Schwann cells double stain with GFAP and S-100 antibodies and are seen to populate each scaffold type equally, demonstrating migration into the scaffold from the animal. eGFP-MSCs are shown to be distributed in close association to blood vessels, in keeping with their function as pericytes. We propose that the tissue formed in MSC scaffold channels is granulation tissue.

The distribution of inflammatory leukocytes, T-cells and microglia is detailed. Microglial cells dominate the channel core area, whereas leukocyte infiltrate is diffuse. Image analysis provides evidence of T-cell immunomodulation in the MSC group. Quantification of axonal counts demonstrates regeneration is augmented by the presence of Schwann cells in implanted scaffolds. MSCs placed in scaffolds do not support axon growth to any extent. Axon regeneration is analysed in relationship to the developing channel vasculature. Methods of unbiased stereology provide insight into physiologic parameters of blood vessels in scaffold channels, derived from estimations of volume fraction, length density, and surface density. Mean vessel diameter and cross sectional area for each channel type are calculated. Whereas Schwann cell channels have high numbers of small, densely packed vessels, infrequent and large vessels dominate the structure of MSC scaffold channels. Significant
correlations between axon counts and vessel length and surface density are shown. Axon number is also shown to statistically correlate with decreasing vessel diameter, implicating the importance of blood flow rate in channels. Radial diffusion distances in vessels correlated significantly to axon number as a hyperbolic function.

In Chapter 4 the development of a retroviral library for gene delivery of neurotrophic factors to Schwann cells and MSCs is described. Retroviral expression plasmids, encoding the cDNA transcripts for human neurotrophin 3 (NT-3), brain derived neurotropic factor (BDNF), and glial derived neurotrophic factor (GDNF), were constructed by molecular cloning. Neurotrophin genes were cloned into the pLXSN backbone, which has been modified to contain an internal ribosomal entry site (IRES) for bicistronic expression of eGFP in target cells. DNA sequence accuracy and eGFP-neurotrophin co-expression were verified prior to the development of GP+E86 packaging cell lines for NT-3, GDNF and BDNF retrovirus production. Neurotrophins are secreted at physiologic levels from target cells following viral infection. Stimulation of neurite outgrowth from dorsal root ganglia is shown in response to conditioned media from target cells infected with NT-3 retrovirus. Stably transduced Schwann cell and MSC lines have been made following retroviral gene transfer and cell selection for use in OPF+ scaffolds.
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Professor Peter Dockery, for his guidance in microscopy and stereology, for the time and resources he dedicated from the Department of Anatomy to the project.

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To members of the REMEDI Lab, who have been a pleasure to work with over many years, Tina, Miriam, Una, Aoiife, Dan, Patrick, Lisa, Eleanor, Alicia, Marc, Aonghus, Sean, and Cathal.

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Dr. Christopher Boes, for putting faith in me and for the opportunities to learn Neurology in such a wonderful place; for all of the support that is required in beginning a combined career as a Clinical Scientist in Neurology.

Dr. Nathan Staff, for his supervision on both the research and clinical front.
Dedication

It is simply not enough to acknowledge Professor Anthony J. Windebank, Professor Timothy O’Brien and Dr. Siobhan McMahon, for their belief in me; for their kindness, support, generosity, mentorship and friendship; in developing and maintaining the project; and in truly forming my career as it stands today. The six years of work done on this project are dedicated to each of them, in recognition of the commitment they have shown to students in their own lives and careers.

With all my love to my family; to my mother Lesley, my father Patrick, my brother Dominic and his family with Christine, Colin and Ellie, to my sister Anna as she begins her own family with Stuart and Capri; to my grandmother Elizabeth and to my sister Brigid.

With all my love to Theresa, as we look forward to our lifetime together.

September, 2011
### Abbreviations

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<tr>
<td>AA2-P</td>
<td>ascorbic acid 2-phosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>BBZ</td>
<td>bis benzamide</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ch-ABC</td>
<td>chondroitinase ABC</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulphate proteoglycan</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3' diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECMV</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
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<td>H&amp;E</td>
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<td>HA</td>
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<tr>
<td>HAMC</td>
<td>hyaluronic acid methylcellulose</td>
</tr>
<tr>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
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<td>human umbilical endothelial cell</td>
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<td>IBA1</td>
<td>ionized calcium binding adaptor molecule 1</td>
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<td>indoleamine 2,3-dioxygenase</td>
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<td>IRES</td>
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<tr>
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<td>Moloney murine leukemia virus</td>
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<td>OPF</td>
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<td>OPF+</td>
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**pHEMA-MMA**  
*pHEMA and pHEMA-co-methyl methacrylate*

**PLGA**  
poly lactic and poly glycolic acid

**PNS**  
peripheral nervous system

**Ppy**  
polypyrrole

**RNA**  
ribonucleic acid

**RT-PCR**  
reverse transcription polymerase chain reaction

**SC**  
Schwann cell

**SCI**  
spinal cord injury

**siRNA**  
short interfering RNA

**SOC**  
super optimal broth

**Sv**  
surface fraction/density

**TAE**  
tris/ acetate/EDTA

**TGFβ**  
transforming growth factor beta

**TH**  
T-helper

**TNFα**  
tumour necrosis factor alpha

**TRITC**  
rhodamine isothiocyanate

**TU**  
transducing units

**V**  
volume

**VEGF**  
vascular endothelium growth factor

**Vv**  
volume fraction
Chapter 1: Introduction

Current Tissue Engineering and Novel Therapeutic Approaches to Axonal Regeneration following Spinal Cord Injury using Polymer Scaffolds

1.1 The Rationale for Tissue Engineering Approaches in the Context of Respiratory Compromise

Current interventions offer little hope of functional recovery for patients after a spinal cord injury (SCI). In the United States, the incidence of SCI is 32 injuries per million population, approximately 11,000 new injuries per year, affecting a young group of people of median age 26, that is predominately male (82%). Road traffic accidents, acts of violence, falls, and sports injuries account for the majority of injuries. The average inpatient stay is 9 months, during and following which the patient’s life, in virtually all aspects, is profoundly changed (Lali, Sekhon et al. 2001; Association 2009). 45.7% of the 253,000 persons living in the United States with the residual of spinal cord injury have permanent and complete paraplegia or tetraplegia, irreversible loss of neurologic function below the level of the injury (National Spinal Cord Injury Association, 2009).

The majority of patients die from respiratory complications. Injury at any level of the spinal cord will impair respiratory function, through the destruction of motor nuclei and descending motor tracts innervating diaphragmatic, thoracic, intercostal and abdominal accessory muscles. Equally impaired are ascending sensory signals for muscle control via stretch reflexes, for cough, vomit and secretion clearance and from peripheral respiratory chemoreceptors. These axons project through the spinal cord to and from a neural network in the brainstem comprising three interconnected centers, the pontine group, and the medullary dorsal and ventral respiratory group. The pontine group (parabrachial/Kölliker-Fuse complex) controls respiratory timing, receives input from lung stretch receptors, and links respiration to behavioural cues; the dorsal group receives
afferents from respiratory chemo and mechanoreceptors, and coordinates respiratory-cardiac reflexes; the ventral group (Bötzinger complex) projects inspiratory neurons, expiratory motor neurons rostrally, and includes a pre-complex generating the respiratory rhythm. Axons descend in the spinal cord in the anterolateral white matter to phrenic, intercostal and abdominal motor neurons, laterally in the high cervical cord near the spinothalamic tract for autonomic function and with the corticospinal tracts for voluntary respiratory control (Nogués and Benarroch, 2008).

Accordingly, respiratory failure with spinal cord injury occurs as a consequence of alternations in tidal volume, ventilation and its pattern, diminished responses to hypercapnia, reduced lung and chest wall compliance, and progressive respiratory muscle fatigue due to compensatory breathing rates. Hypoxia from respiratory compromise can further the neurologic injury. Common secondary pathology includes (aspiration) pneumonia, atelectasis and the complications of mechanical ventilation (Lane et al, 2008). Injury to the cord can also induce paralytic ileus worsening aspiration. More severe respiratory compromise occurs with higher levels of injury with risk to phrenic motor nuclei located in cervical spinal cord segments C3-C5 (occasionally as low as C7) (Zimmer et al., 2007).

Pathological (Quencer and Bunge 1996) and imaging studies (Bodley 2002) demonstrate tissue destruction with cysts and gliosis in the area of injury, along with atrophy in adjacent segments of cord. Strategies aimed at preventing immediate and delayed secondary damage need to be administered within minutes or hours of injury. Even if ideal protective agents were available, many patients would not be in circumstances where this would be available or successful. The area of cysts and glial scarring does not contain cells or tissue that contribute to regeneration and is consequently both a gap and a barrier to regeneration. There are, therefore, only two ways to re-establish neurologic function below the block: bypassing the area or rebuilding functional tissue within the cyst/scar. A functional bypass might be established by nerve autograft connections from areas above the lesion to distal effectors (cord or muscle) (Tadie, Liu et al. 2002). The second approach is to replace the cyst/scar with functional tissue, promoting the development of neural tissue bridges to carry regenerating axons from above to roots or muscles below the lesion (Friedman, Windebank et al. 2002). For future
use in patients, replacement of a segment of cord would be suitable for those with massive damage to the cord with no evidence of residual functional tissue in the area. Unfortunately, this accounts for a significant number of patients.

Animal models of spinal cord injury include complete transection (thoracic), hemisection (dorsal or unilateral), and contusion injuries (forceps and computer-controlled weight impact). These models approximate common human pathology, open cord laceration (1/4 of injuries) and closed compression/contusion injuries (3/4). Biomaterial polymers may be delivered as gels, suitable for contusion and small tears, as devices designed to fill larger defects (sponges) or to bridge large gaps and traverse the glial scar (tubes and multichannel scaffolds (Nomura, Tator et al. 2006). While deep tears or transections are rare in human injury, complete or partial transections in animal models are useful as proof of concept, and for the controlled study of axonal regeneration (Talac, Friedman et al. 2004). Animal models of respiratory dysfunction focus on high cervical injury producing diaphragm hemiplegia, but no studies to date have employed polymer-based tissue engineering strategies specifically in this context. Given the severity of patient morbidity and the rates of mortality associated with respiratory compromise, neurologic repair is an important therapeutic goal. A relatively short distance, from the medulla to phrenic C3-C5 or within the phrenic segments for example, needs be bridged by new neuronal tissue. Equally, respiratory innervation associates with discrete tracts, corticospinal and spinothalamic, and repair may often be unilateral given a lateral injury and diaphragmatic hemiplegia. Such tracts represent ideal targets for polymer scaffold implantation given their limited scope and clinical importance.

1.2 Important Bioengineering Considerations

The classification of biomaterials for the spinal cord is based on whether the materials are naturally derived or synthetic, whether they are hydrogels, whether or not they are biodegradable, as well as other sub-classifications based on specific modification or functional adaptation (surface charged, drug-delivery etc.). Regardless of the source or application, the material must have properties which are compatible within the spinal cord environment (Kohane and Langer
These properties in turn influence the regenerative capacity of engineered structural support for neurite outgrowth at a macro (i.e. fascicular) and micro (axonal) level.

### 1.2.1 Scaffold Placement within the Spinal Cord Environment

In relation to blood, cerebral spinal fluid is low in cellular nutrients. Scaffold permeability to various molecular sizes becomes crucial for access to oxygen and nutrients and removal of metabolic wastes. The degree to which a material swells within the aqueous environment of the spinal cord must be known if the scaffold is to maintain an appropriate alignment and not compress regenerating nerves.

Degradation kinetics may be accelerated by the ingrowth of axons and by the deposition of extracellular matrix by support cells of the CNS or by the therapeutic cell line seeded within the scaffold. Stiffness, permeability, swelling, strength and degradation are of course specific to the particular polymer employed, are all readily modified through changes in polymer concentration or constituent ratios. (de Ruiter, Onyeneho et al. 2008) exemplify the type of in vitro characterization and methodology required to develop an implant of any polymer type. In this study,

**Figure 1.1:** Polymer scaffold in situ. Lateral radiograph showing a well aligned scaffold within the spinal canal after 4 weeks. The spine has been fixed and the scaffold contains barium contrast within the polymer. From (Rooney, Vaishya et al. 2008) with permission.
the authors present a series of methods to characterize multichannel nerve tubes for properties of bending, deformation, swelling, and degradation and introduce a new method to test the permeability of multichannel nerve tubes from the rate of diffusion of different-sized fluorescent dextran molecules. Equally, the implantation methodology must be developed. While the material is placed within rigid spinal column, the spine may require further fixation (Figure 1.1) (Rooney, Vaishya et al. 2008). Scaffolds in unfixed spines have a greater tendency to produce scoliosis and become displaced (Figure 1.2). The material should be of sufficient softness not to physically damage the cord as the animal moves. The degradation products of the polymer, and any residual agents used in its fabrication, cannot be locally or systemically cytotoxic or elicit an immune response which will further gliosis, and be destructive both to the scaffold complex and any regenerating axons (Liu and Cao 2007).

Cells detect mechanical characteristics of the environment through adhesion complexes and the actin cytoskeleton, and the stiffness of the substrate may be of critical importance (Discher, Janmey et al. 2005). Finally the tensile strength of the material – its ability to hold a suture for example, will contribute to its clinical use.
1.2.2 Material Fabrication and Porosity

Scaffold biomaterials for spinal cord placement are fabricated by dissolving the monomer in an aqueous or organic solvent to produce a liquid state that is polymerized into macromers by a chemical, thermal or photo-crosslinking reaction. Additional reagents may be added to enhance the crosslinking reaction. Synthetic polymers often employ the use of chemical initiators and accelerators to fine tune polymerization rates. The majority of spinal cord scaffolds are made by injection molding. To create pores in the structure, porogens are incorporated into the polymer mix. Commonly used porogens include sodium chloride crystals, ice crystals, gas bubbles introduced by peroxides or air-foaming, and gelatin composite materials. In each case, the polymer forms around the porogen, which

Figure 1.2: The effect of spine stabilization on scaffold alignment. 3-dimensional magnetic resonance microscopy (MRM) in coronal (A and B) and axial (C and D) images 4 weeks after scaffold placement into the transected cord with (A and C) and without (B and D) spine fixation. From (Rooney, Vaishya et al. 2008) with permission.
itself is removed from the final structure leaving only the space it occupied (Figure 1.3). The size of the pore therefore can be controlled by adjusting the variables that control crystal size and the direction of crystal growth (Madaghiele, Sannino et al. 2008), or by adjusting the water content of a hydrogel.

A continuous porous structure closely mimicking the intrinsic mechanical characteristics of the original tissue may provide a better environment for regeneration (Discher, Janmey et al. 2005; Deguchi, Tsuru et al. 2006). Material porosity is essential for cell attachments, allows for greater distances that may be bridged, and improves functional recovery following transection (Jenq and Coggeshall 1987; Vleggeert-Lankamp, de Ruiter et al. 2007; Reynolds, Bren et al. 2008). Porosity also allows for tissue vascularization of the avascular scaffold.
Implant, influences cell migration and phenotype, and will improve implant stability at the cord-scaffold interface through interlocking between implant and cord tissue (Dadsetan, Hefferan et al. 2008).

1.2.3 Macroengineering

Nerve tracts in the human spinal cord have diameters of 100–1000 \( \mu \text{m} \). It is the role of scaffold macro-engineering to design conduits whose architecture optimizes axonal growth potential through the alignment of fascicular groups (Figure 1.4). Channel sizes depicted in the templated agarose scaffold are on the order of 200 \( \mu \text{m} \) (Figure 1.4A), and 450 \( \mu \text{m} \) in the PLGA scaffold (Figure 1.4C).

**Figure 1.4:** Macroarchitectural Design. Various injection-molding strategies for spinal cord scaffolds to align fascicular bundles. (A) Templated agarose cast over polystyrene fibres produces linear aligned channels of 200 \( \mu \text{m} \) diameter for a hemisection model (from (Stokols, Sakamoto et al. 2006) with permission. (B) Cylinder, tube, multichannel and open-path designs, from (Wong, Leveque et al. 2008) with permission, for casting in poly (\( \epsilon \)-caprolactone). (C) PLGA multichannel scaffolds cast over parallel metal wiring provide dorsal and ventral channels, (left, bar 500 \( \mu \text{m} \), from (Moore, Friedman et al. 2006) which may be elaborated into molds of complex anatomical design, (right, (Friedman, Windebank et al. 2002) both figures with permission).
In the first study of its kind, Wong et al (2008) directly compared porous poly (ε-caprolactone) synthetic polymer scaffolds cast in five different architectures, cylinder, tube, multichannel, and open-path design with and without a central core (Figure 1.4B). The findings demonstrated not only that open path designs were improvements over the other three designs in terms of regenerative capacity, but also that the other more closed designs adversely affected the surrounding cord, doubling the defect length. In a multichannel model, the channel size is of importance. Spinal cord scaffolds with multiple longitudinally-aligned channels of 450 and 660 µm were constructed from PLGA using injection molding. When seeded with Schwann cells, this scaffold design supported robust axonal growth (Moore, Friedman et al. 2006), although also demonstrated the formation of a rim of fibrous tissue surrounding the core of regenerated neurons. Further work evaluating the relationship between scaffold channel diameter and the number of axons regenerating showed a larger area of fibrous tissue and a reduced axon number in the larger channel size (Krych, Rooney et al. 2009).

1.2.4 Microengineering

A large myelinated axon in the CNS has a diameter of 15 – 20 µm. In order to create functional reconnections, the scaffold must also regionally align separated neuron groups at an axonal and growth cone level. Scaffold microengineering refers to designing features that are in the order of a few microns in at least one dimension (Khademhosseini and Langer 2007). In the context of the spinal cord, this includes micro-structures that will provide precise directionality to growth and improve axonal adherence at the level of the advancing growth cone. It is conceptually useful to consider the re-alignment of nerve fascicles as three dimensional bundles of axons which themselves prefer to grow or elongate along the planes of two dimensions (Bellamkonda 2006). Microgrooves can be placed in the polymer surface by laser etching, affecting contact guidance and alignment of neurites. In vitro work has demonstrated optimal sizes of 2 µm minimum groove depth (Clark, Connolly et al. 1991), with more narrow ridges (5 µm versus 10 µm) improving the number neurites aligned as well as the number of focal contact adhesions in a given cell (Goldner, Bruder et al. 2006). Further improvements in neurite outgrowth are seen with coating the
grooved surface with collagen or laminin peptides, Figure 1.5 (A and B) (Yao, Damodaran et al. 2010). Axonal growth cones preferentially advance up gradients of laminin (Adams, Kao et al. 2005). For the spinal cord, gradients have been made in natural and synthetic polymers with laminin (Dodla and Bellamkonda 2006).

**Figure 1.5.** Oriented axonal growth. Microengineering strategies with oriented extension of neurite outgrowth in relation to micropatterned grooves and nanofibres. SEM imaging of PC12 cell growth on a collagen type I-coated PLGA film (A) and on a laminin peptide-coated PLGA film (B) each with a laser-etched groove size of 10 μm. Bar 40 μm, from (Yao, Wang et al. 2009). Neurite extension along the length of electrospun PCL fibres of a diameter ranging from 0.8 +/- 0.7 μm (C) and 3.7 +/- 0.5 μm (D), from (Yao, O'Brien et al. 2009) with permission.
Axons also show a preference to grow along the length of micro and nano-fibres of polymers such as collagen and synthetic polymers, Figure 1.5 (C and D). Polymer fibres are made by monomer self-assembly, producing a randomized fibre orientation of larger caliber fibres, or by electrospinning for parallel alignments of nanometer scale fibres (50 nm – 30 µm). This technique uses electric charge to draw and elongate threads of liquid polymer (collagen, PLGA, PCL) from a syringe pump source. The solvent evaporates from the airborne filament which is laid down upon an electrically grounded plate or rod, or onto a spindle apparatus (Yang, De Laporte et al. 2005). Collagen filament bridges with fibres of 20 µm show promise as 2 dimensional surfaces to guide axons, improving axonal density and motor function, but appear also to have significant cytotoxicity with high animal mortality in a rabbit model (Yoshii, Oka et al. 2004; Yoshii, Ito et al. 2009). A goal of scaffold microengineering is to pack larger channels of scaffolds with these fibrous substrates to approximate normal axonal densities (Schnell, Klinkhammer et al. 2007).
### Table 1.1

The major natural polymers in use in animal models of spinal cord injury and examples of their applications

<table>
<thead>
<tr>
<th>Natural Polymers</th>
<th>Structure</th>
<th>Examples of Applications for Spinal Cord Repair</th>
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<tr>
<td><strong>Natural Hydrogels</strong></td>
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<tr>
<td>Hyaluronic acid</td>
<td>Dissacharide units of glucuronic acid and N-acetylglucosamine</td>
<td>Intrathecal drug and growth factor delivery with as a methylcellulose composite gel. (Gupta, Tator et al. 2006; Shoichet, Tator et al. 2007; Kang, Poon et al. 2009)</td>
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1.3 Biomaterials for the Spinal Cord

1.3.1 Natural Polymers

The spinal cord lacks a support matrix equivalent to the endoneurium and perineurium in peripheral nerve, one that can act as conduits to approximate disconnected axonal groups. Furthermore the axonal density of the spinal cord far surpasses that of peripheral nerve with far less extracellular matrix support. The rationale for polymer implants is to replace a damaged area of the cord with just such a structural matrix. Natural polymers are biological fibrillar protein, polysaccharide, or glycosylaminoglycan (GAG) carbohydrates which form hydrogels. These polymers already have an intrinsic function such as extracellular matrix or structural support, and a degradation profile by \textit{in vivo} enzymes that is in keeping with their natural role. Hydrogels are mesh networks of insoluble polymer fibres, through which water can freely flow to osmotically swell or shrink the overall structure. Hydrogels are attractive materials for use in the spinal cord. They are macroporous, soft materials readily allowing cell adhesion and migration, while nutrients and wastes are easily exchanged. They can be easily shaped to fit the defect, their elasticity and degradation may be adjusted by component density. Table 1 details the main natural polymers and highlights examples of their application within the spinal cord. Two spinal cord extracellular matrix (ECM) components are used, collagen and hyaluronic acid (HA); two polymers are derived from marine plants, agarose and alginate, and chitosan is derived from insect or crustacean shells.

\textit{Extracellular matrix-based polymeric systems}

Being the predominant extracellular matrix protein, type I collagen has intrinsic properties including molecular sites for cell adhesion and migration, inherent signalling transduction for proliferation and differentiation, and mechanical properties similar to soft tissue. Antigenicity is low, provided the origin species is the same as the host. Solutions of collagen are polymerized by adjusting pH, or with the addition of ionic salts. Whereas early use focused on its application as a three dimensional matrix growth, it was soon realized that collagen itself has a limited capacity to support axon growth (Marchand, Woerly et al. 1993), and that its use required further functionalization. An important trend
for the use of collagen is in combined strategies, particularly as a growth or elution matrix within or on the surface of other polymer types (Tsai, Dalton et al. 2006) or as a composite material (Cheng, Deng et al. 2003). Axonal extension onto collagen can be improved through covalent modification, or the incorporation of cell-adhesion molecules such as laminin, to provide directional guidance as a gradient along collagen fibres (Yao, O'Brien et al. 2009). Collagen also has the advantage of being thermoresponsive, gelling at physiologic temperatures, making it attractive for use as an injectible polymer delivery system. This property enables the incorporation of neurotrophic factors, drugs or cells at the time of gelation without thermal damage to the factor or cell line. Incorporation of neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) (Houweling, Lankhorst et al. 1998) improved axonal counts and animal function, including a specific regional improvement of corticospinal tract density with the use of NT-3. Degradation of the collagen in situ allows for sustained release of the growth factor, as well as an improved surface for cell attachments.

The glycosaminoglycan HA was thought to be a good material candidate given its role as extracellular matrix in the brain, but its success in supporting axonal growth is modest. As a scaffold material, HA has been used with benefit as a matrix for cultured embryonic spinal cord tissue placed into transected cord (Rochkind, Shahar et al. 2002). HA has however been developed into an extremely useful co-polymer gel with methylcellulose for intrathecal drug delivery (Gupta, Tator et al. 2006). Whereas methylcellulose gels at increasing temperature, unmodified HA quickly disperses in vivo. The combination of acetate-modified HA with methylcellulose (HAMC) has the distinctive property of already gelling at room and physiologic temperature prior to its delivery, but become liquid when subjected to the mechanical shear forces involved with syringe and needle injection (Katz and Burdick 2009). Collagen embedded epidermal growth factor (EGF) (Shoichet, Tator et al. 2007), and erythropoietin (Kang, Poon et al. 2009), have been safely delivered with sustained release in situ from HAMC. The latter agent enhanced neuroprotection with reduced cavitation size and increased neuron numbers following clip compression of the spinal cord.
Polymers extracted from marine life

Agarose is used in many of the same ways as collagen for spinal cord repair. It is a linear polysaccharide derived from seaweed and cross-linked by temperature gradients through hydrogen bonding. Agarose is thermoresponsive, but at temperatures lower than 37°C. It has been used as an injectable system when it can be rapidly cooled in situ using liquid nitrogen vapour (Jain, Kim et al. 2006). Such a system is now being developed for direct topical delivery of dexamethasone onto the injury site, from drug eluting nanoparticles suspended within an agarose implant (Chvatal, Kim et al. 2008). Early inflammatory infiltrates and lesion size were reduced by day 7. Like collagen, agarose itself is relatively impenetrable by axons, but serves as an excellent axonal growth substrate, particularly when functionalized with laminin gradients (Dodla and Bellamkonda 2006). Tuszynski and colleagues have used a freeze drying method to form agarose scaffolds containing linear guidance pores with a mean diameter of 120 µm (Stokols and Tuszynski 2004; Stokols and Tuszynski 2006). This process involves the formation of ice crystals whose size and direction of growth can be controlled by the temperature gradient (Tabesh, Amoabediny et al. 2009). Pore size in the scaffold can also controlled by the freezing rate and pH, with the faster rate creating smaller sizes (Sachlos and Czernuszka 2003). Integrating BDNF into the scaffold material (Figure 1.6) and in separate experiments, BDNF-secreting mesenchymal stem cells scaffold channels, significantly improved the scaffold’s capacity to promote regeneration (Stokols and Tuszynski, 2006). BDNF within lipid microtubules has also been incorporated into agarose scaffolds, enhancing axonal growth for the length of the scaffold but not into the distal cord (Jain, Kim et al. 2006).

Alginate is obtained from algae and the polymer solution is crosslinked by calcium into a sponge-like structure. Such a structure supported axonal extension in the spinal cord and limited gliosis (Kataoka, Suzuki et al. 2001). Hippocampal neurospheres and BDNF-secreting fibroblasts have been seeded onto alginate and placed into the transected cord (Nomura, Katayama et al. 2006). Agarose and alginate requires ultrapurification prior to use, given that commercial preparations often contain mitogens and cytotoxic byproducts.

Chitosan is a glycosaminoglycan polymer derived by chemical deacetylation of chitin, the major structural polysaccharide found in crustacean,
shellfish and insect shells. Cell adhesion to the structure is determined by the extent of its positive charge, itself a function of the degree of alkaline deacetylation (Nisbet, Crompton et al. 2008). Further improvements in cell attachment are seen with the addition of poly-L-lysine to the polymer mix, and a thermoresponsive polymer can be made with the addition of glycerol phosphate salts (Crompton, Goud et al. 2007). Chitosan scaffolds support axonal growth in the spinal cord (Freier, Montenegro et al. 2005), and the polymer may be used to encapsulate therapeutic cell lines (Yuan, Zhang et al. 2004), Figure 1.7 (lower). Recent work by Shoicet and colleagues demonstrates chitosan’s use as extramedullary and intramedullary conduits capable of supporting neural stem cell differentiation in the transected cord (Nomura, Zahir et al. 2008; Zahir, Nomura et al. 2008).

**Polymers derived from the blood**

Plasma derived polymers, fibronectin and fibrin, are being used as spinal cord scaffolds. Fibronectin mats are formed with linearly aligned fibres which can orient axonal growth (King, Henseler et al. 2003) and sequester growth factor within its pores for gradual release (Phillips, King et al. 2004). Fibrin scaffolds are formed from monomers following fibrinogen cleavage by thrombin and crosslinked with Factor XIIIa (Willerth and Sakiyama-Elbert 2007). It is a natural matrix for wound repair having inherent cell-binding sites. Heparin has been crosslinked to fibrin scaffolds using a bidomain Factor XIIIa-heparin linker peptide for use as an affinity-based delivery system for growth factors, including NT-3 (Taylor and Sakiyama-Elbert 2006) and for factors to differentiate embryonic stem cells seeded within the matrix. A recent study implanting fibrin polymer scaffolds into a dorsal hemisection model demonstrated delayed astrocytosis and improved neuron fibre extension (Johnson, Parker et al. 2009), Figure 1.7 (upper panels).
Figure 1.6: Freeze Dried Agarose Scaffolds in a complete transection model, figure from (Stokols and Tuszynski 2006) with permission. Upper Panel: Scanning electron microscopic images of scaffolds in (A) longitudinal or (B) cross-sectional orientation shows the arrangement of channels in a honeycomb structure. Scale bar is 100 μm. Lower Panel: Neurofilament labeling demonstrates penetration and linear growth of axons within channels of scaffolds. (A) Scaffold lacking growth factor. (B) Scaffold loaded with 2 μg recombinant human BDNF into walls and matrix-filled lumen of individual channels. Magnitude of linear axonal growth is significantly increased. (C) Best example of linear axonal growth through complete length of channel. Scale bars=100 μm.
Figure 1.7: Scaffolds in situ. A dorsal hemisection injury (A) is filled with a fibrin matrix, as visualized with fibrinogen immuno-fluorescence (B). Diverse applications have been developed with this polymer, including affinity-based drug elution from heparin complexes and embryonic stem cell differentiation. Bar is 200 µm. Adapted from (Johnson, Parker et al. 2009) with permission. (C-F) A complete spinal cord transection is bridged with chitosan scaffolds loaded with brain-derived neural stem cells (C and D) or Schwann cells (E and F) as seen from a dorsal and lateral aspect. Neuronal tissue bridges have developed from the transected cord stumps. (Zahir, Nomura et al. 2008) with permission.
1.3.2 Synthetic Polymers

Whereas the use of natural polymers in the spinal cord takes advantage of their inherent properties, their natural role to some extent being the basis of their function or their modification, using synthetic polymers offers wider scope to design and control the characteristics of the material. The synthetic polymers used thus far in the spinal cord are either biodegradable materials based around polyesters of lactic and glycolic acid (PLA and PGA), are biodegradable hydrogels based on polyethylene glycol (PEG), or are non-biodegradable hydrogels based on methacrylate. Early spinal cord scaffolds were made from the same materials as were in common clinical use for surgical repair of peripheral nerve and skin grafting. Rapid advances in hydrogel chemistry have produced materials more suited to the spinal cord in their mechanical properties. The trend now is towards using these highly aqueous, soft polymers given the similarity of their properties to spinal cord tissue, and the versatility with which their chemistry and architecture can be adjusted. Functionalized synthetic polymers have included gradients and surface charge modification for cell adhesion, neurotrophic gradients, and have opened the field to using scaffolds themselves as drug delivery and gene delivery vehicles to novel extent. The technical ability to rapidly photocross-link synthetic hydrogels has also enabled a remarkable degree of sophistication in macro and micro-architecture through the use of photolithography. Please see Table 2 for a listing of synthetic polymers and examples of their use within the spinal cord.

Poly α-hydroxy acid polymers

At the time of our previous review (Friedman, Windebank et al. 2002), much of the work in scaffold design focused on the use of biodegradable synthetic polymers, particularly the poly (α-hydroxy acids) PLA, PGA and their copolymer PLGA. These polymers are polyester links of lactic and glycolic acid which are hydrolyzed in vivo to release lactide and glycolide, dissolving the material. The pH around the grafted site accordingly will become more acidic. These compounds were a good initial choice for spinal cord placement, having a long track record of FDA approved clinical use as an absorbable suture material, and as grafting material for skin and for peripheral nerve repair (Mackinnon and Dellon 1990; den Dunnen, Van Der Lei et al. 1993). The transition to CNS...
applications was based on the idea that such scaffolds could provide a corresponding PNS-like endoneurial and perineurial guidance structure to regenerating spinal cord axons, improved with the addition of myelinating Schwann cells within the scaffold (Hadlock, Sundback et al. 2000).
Table 1.2
The main synthetic polymers in use in animal models of spinal cord injury and examples of their applications.

<table>
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<th>Synthetic Polymers</th>
<th>Structure</th>
<th>Examples of Applications for Spinal Cord Repair</th>
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<tbody>
<tr>
<td><strong>Biodegradable</strong></td>
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<tr>
<td>PLA</td>
<td>Poly(D,L lactic acid)</td>
<td>Single and multichannel scaffolds seeded with Schwann Cells.</td>
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<td>PGA</td>
<td>Poly(glycolic acid)</td>
<td>PLA: Single channel (Oudega, Gautier et al. 2001)</td>
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<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
<td>Freeze dried macroporous foam scaffold with incorporated BDNF (Patist, Mulder et al. 2004)</td>
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<td></td>
<td>Freeze dried macroporous scaffold with BDNF-secreting Schwann cells (Hurtado, Moon et al. 2006)</td>
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<td>PLGA: Multichannel scaffolds seeded with Schwann cells (Moore, Friedman et al. 2006)</td>
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<td>Multichannel scaffolds seeded with neural stem cells (Teng, Lavik et al. 2002)</td>
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<td>Neurotrophin-eluting micro and nanospheres within scaffolds (Yang, Murugan et al. 2005; Wang, Wu et al. 2008)</td>
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<td>Protein release from chitosan scaffolds (Kim, Tator et al. 2008)</td>
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<td>PCL</td>
<td>Poly-ε-caprolactone</td>
<td>Nanofibre spinning for axonal growth orientation (Schnell, Klinkhammer et al. 2007)</td>
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<td>PCLF</td>
<td>Poly-ε-caprolactone fumarate</td>
<td>Multichannel scaffolds (Wang, Mullins et al. 2009)</td>
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<td><strong>Hydrogels</strong></td>
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<td>PEG</td>
<td>Polyethylene Glycol</td>
<td>Intravenous solution (Laverty, Leskovar et al. 2004)</td>
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<td>Immunoprotective sealant gel (Borgens, Shi et al. 2002; Duerstock and Borgens 2002)</td>
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<td>Injectable gel with PLA for NT-3 delivery (Piantino, Burdick et al. 2006) and BDNF (Soderquist, Milligan et al. 2008)</td>
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<td>3-Dimensional neurite growth matrix (Namba, Cole et al. 2009)</td>
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<td>Intravenous PEG with Magnesium Sulfate (Ditor, John et al. 2007; Kwon, Roy et al. 2009)</td>
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<td><strong>Non-biodegradable hydrogels</strong></td>
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<tr>
<td>pHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
<td>pHEMA sponges (Giannetti, Lauretti et al. 2001)</td>
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<td>Guidance channels (Tsai, Dalton et al. 2004)</td>
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<td>Fibre templated scaffolds (Flynn, Dalton et al. 2003)</td>
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<td>Cell adhesion gradients (Yu and Shoichet 2005)</td>
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<td>Neurotrophic gradients (Moore, MacSween et al. 2006)</td>
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<td>Co-polymer with chitosan including cell adhesion peptides (Yu, Kazazian et al. 2007)</td>
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<td>Coil (Nomura, Katayama et al. 2006) or multilayered pHEMA (Carone and Hasenwinkel 2006) scaffold reinforcement</td>
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<td>Surface charge modification for cell adhesion (Lesny, Pradny et al. 2006) and axonal growth (Hejcl, Urdzikova et al. 2008)</td>
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<td>Matrix filled channels for acidic fibroblast growth factor (FGF-1) and NT-3 delivery (Tsai, Dalton et al. 2006)</td>
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<td>Colloid gel with cell adhesion peptides (Woerly, Pinet et al. 2001) (Neurogel™)</td>
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<td>Colloid gel with CNTF and BDNF (Loh, Woerly et al. 2001)</td>
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<td>PAN/PVC</td>
<td>Poly(acrylonitrile-co-vinylchloride)</td>
<td>Schwann cell seeded scaffolds with BNDF &amp; NT-3 cord infusion (Bamber, Li et al. 2001)</td>
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<td>Schwann cell seeded scaffolds with GDNF Matrigel™ (Iannotti, Li et al. 2003)</td>
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PLA has been used to make scaffolds. Having shown the resorbability and biocompatibility of PLA with Schwann cells and the spinal cord (Gautier, Oudega et al. 1998), the University of Miami group placed single channel Schwann cell loaded scaffolds into the transected rat spinal cord. The PLA materials used were structurally unstable, fragmenting and collapsing, but proved to support the extension of axons and vascular growth into the graft (Oudega, Gautier et al. 2001). More recently, macroporous PLA foam scaffolds made with longitudinally aligned pores were fabricated using a freeze drying technique. BDNF was dissolved into the scaffold matrix but did not improve an overall low yield in axon numbers through the graft (Patist, Mulder et al. 2004). This study was extended to incorporate Schwann cells into the foam that had been genetically engineered to secrete a bi-functional neurotrophin (D15A) with BDNF and NT-3 activity (Hurtado, Moon et al. 2006). Axonal regeneration was modest at 6 weeks, and few Schwann cells survived the first week of scaffold placement. There has been recent interest in the use of PLA nanofibres as a cell substrate (Wang, Mullins et al. 2009).

The degradation rate of PLA can be somewhat controlled in the co-polymer PLGA by altering the ratios of the PLA and PGA in composite. The \textit{in vitro} characteristics (bending, swelling, deformation, degradation and permeability) of varying PLA:PGA ratios have been extensively determined for PLGA spinal cord scaffolds implants (de Ruiter, Oynekeho et al. 2008). Multichannel PLGA scaffolds have been demonstrated to support robust axonal regeneration when seeded with Schwann cells without functional improvements (Moore, Friedman et al. 2006). As detailed in the section below, PLGA degradation kinetics are the basis for drug delivery via microspheres embedded in polymer scaffolds.

\textbf{Synthetic hydrogels}

Polyethylene glycol is a biodegradable synthetic polymer of ethylene oxide units. Its role is somewhat unique in that it is an ‘exclusionary’ compound, immunoprotecting the areas to which it is applied by keeping out cell infiltrates, and equally is used as a delivery system for cells, neurotrophins and genetic constructs. It has been formulated into gels for topical application onto the injured
Axonal Regeneration Supported by OPF+ Cell-loaded Hydrogel Scaffolds

spinal cord (Borgens, Shi et al. 2002), combined with PLA for neurotrophin delivery of a photo-inducible gel, or as a formulation of ‘pegylated’ BDNF as an intrathecal infusion (Soderquist, Milligan et al. 2008). Intravenous solutions with magnesium sulfate are being investigated as a first line immunomodulatory therapy in acute spinal cord injury (Kwon, Roy et al. 2009).

Poly(2-hydroxyethyl methacrylate) (pHEMA) polymeric systems were initially used as non-biodegradable materials for soft contact lenses. Professor Shoicet’s group in Toronto has extensively developed pHEMA and pHEMA-co-methyl methacrylate (pHEMA-MMA) for use as spinal cord scaffolds. Early applications included pHEMA sponges (Giannetti, Lauretti et al. 2001), evolving to guidance channels (Tsai, Dalton et al. 2004), with a variety of surface modifications to improve cell adherence and axonal extension (Moore, MacSween et al. 2006; Tsai, Dalton et al. 2006) including neurotrophic gradients and adhesion gradients within a chitosan composite (Yu, Kazazian et al. 2007). Microfluidic techniques are employed to create functional concentration gradients in scaffolds, again providing cues for directionality of axonal growth. The technique involves casting a scaffold using 2 or more inlet ports coupled with rapid polymerization. An important advantage of this polymer class is the modification of surface charge with the addition of quaternary amine groups or of a second methacrylate subtype. Many cell types adhere better to a positively charged surface. Lesny et al. (2006) evaluated the in growth of neural tissue in a dorsal hemisection injury bridged with four pHEMA composites, demonstrating an improvement in axonal regeneration into the core of the scaffold and a reduction in astrocyte infiltration in the positively charged scaffold.

Very sophisticated architecture is possible with these compounds. The scaffolds have been reinforced with coils, as well as being made as with neurotrophin PLGA microspheres. Multi-layered macroporous pHEMA composites, including innermost channel layers that elute neurotrophic factors, have been made by liquid-liquid centrifugal casting. Thin layers of the polymer liquid are forced against the side of the mold by the centrifugal force and polymerized. The polymers can also be crosslinked with light with the addition of a photoinitiator. Using photolithography, in which discrete sections of the polymer mix are exposed to light while others are masked, open channel size and
pore size was recently controlled with remarkable precision (Bryant, Cuy et al. 2007).

1.4 Biomaterials as Therapeutic Cell Systems in the Spinal Cord

As one of the field’s principal contributors, Bellamkonda advocates that scaffold strategies for nerve regeneration should combine four main components, a permissive growth substrate (hydrogel or micro/nano fibre), a neurostimulatory extracellular matrix (protein or peptide), the provision of neurotrophic factors, and glial or support cells (Schwann cells, neural stem cells). Cell therapies can be delivered to the spinal cord by direct injection into the cord substance, intrathecal infusion, or by polymeric microspheres or scaffolds. It has been shown that a variety of cells support axonal regeneration within polymer scaffold models in the cord. Cells can be loaded onto polymer scaffolds when suspended in a support matrix such as fibrin or Matrigel™. If the scaffold is a macroporous hydrogel, the cells migrate and become resident within that porous structure, and if the scaffold is designed to be multichannel, select channels can be seeded with different cell types allowing for regional topography (Friedman, Windebank et al. 2002).

1.4.1 Schwann Cells

Early work in the spinal cord was again derived from peripheral nerve repair strategies. Schwann cells myelinate peripheral nerve, and will naturally migrate from peripheral-central nerve junctions such as the dorsal root ganglia during times of cord injury to support repair (Oudega, Moon et al. 2005). Here they play a structural role, lay down extracellular matrix proteins like laminin, and provide paracrine trophic support through secretion of nerve growth factor (NGF), NT-3, BDNF, ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) (Willerth and Sakiyama-Elbert 2008). Xu et al (1995) placed Schwann cells expanded in culture from harvested rat sciatic nerve into a poly(acrylonitrile-co-vinylchloride) (PAN/PVC) nerve conduit and into a complete cord transection model. The study demonstrated axonal extension and myelination of about a quarter of the regenerated sensory nerves as seen with
electron microscopy, and that the extent of the repair could be enhanced by administering methylprednisolone in the acute phase of the injury (Chen, Xu et al. 1996). The Miami group also utilized peripheral nerve grafts placed directly into the transected spinal cord serving as natural conduits for axonal extension and as a source of Schwann cells (Oudega and Hagg 1996; Oudega and Hagg 1999). This work was extended to cell-seeding polymer scaffolds, demonstrating evoked nerve conduction potentials across the graft only in the Schwann cell group (Pinzon, Calancie et al. 2001). In all applications, it is important to understand whether cells survive in the grafted material (natural or synthetic). We have demonstrated cell survival for up to 6 weeks after transplantation through a multichannel scaffold, and that in this model, Schwann cells have a higher capacity than stem cell neurospheres to enhance axonal regeneration in the transected cord, Figure 1.8 (Olson, Rooney et al. 2009).
Figure 1.8: Neurofilament staining of axons in transverse sections through a PLGA scaffold after 1 month in vivo. Fluorescent microscopy image of one transverse section of a scaffold (loaded with NSCs) stained with an antibody against neurofilament with the region in which axons were counted encircled by a white line (A). Transverse sections of one channel from the NSC group (B), one from the SC
1.4.2 Olfactory Ensheathing Cells (OECs)

Olfactory ensheathing glia are cells within the peripheral and central component of the olfactory system that contribute to the regenerative capacity of olfactory neurons. The average lifespan of olfactory receptor neurons is four weeks. These neurons are bipolar cells projecting to the nasal epithelium and to the olfactory bulb. New receptor neurons are derived from a stem cell layer at the base of the nasal epithelium, from where axons are projected through the cribriform plates into olfactory bulbs. The role of OEC is to enfold and guide growing axons to the bulb in bundles of unmyelinated axons, and once at the bulb to interact with resident astrocytes and fibroblasts to finalize the connections (Franssen, de Bree et al. 2007). While extensive work (over 40 in vivo studies) has been done with cell injections into the cord, few studies have utilized polymer scaffolds. In one of the first and most successful studies Bunge’s group used Schwann-cell seeded PAN/PVC conduits in a thoracic transection model, now in combination with stereotactic injection of OEC’s at four midline depths. The OEC’s induced axonal growth in through the SC-containing channel and for distances of 2.5 cm, the longest distance observed thus far for OECs (Ramon-Cueto, Plant et al. 1998). Followup studies showed functional recovery without the use of scaffolds (Ramon-Cueto, Cordero et al. 2000). Lu and Ashwell (2002) used collagen matrix soaked with OECs to bridge a dorsal transection and with similar efficacy to pieces of olfactory lamina propria and OEC injections. Chuah et al (2004) encapsulated OEC’s into polyvinylidene fluoride particles for injection following dorsal transection which increased the numbers of collateral branches from the intact ventral cord. A strategy which used Schwann cells embedded in a Matrigel™ bridge, injections of OEC’s into the distal and proximal cord stumps following transection, and alternate day delivery of Chondroitinase...
ABC via an intrathecal catheter, resulted in select fibre regeneration and some recovery of function (Fouad, Schnell et al. 2005).

### 1.4.3 Neural Stem Cells

Neural Stem Cells (NSCs) are a pluripotent, self-renewing population of precursor cells that give rise to astrocytes, oligodendrocytes, and neurons in the CNS. Their role in regenerative medicine is therefore to remyelinate axons growing from the injured cord, to themselves become neuronal links within the injury (Lowry and Temple 2007), and to elaborate neurotrophic factors to stimulate regrowth (Lu, Jones et al. 2003). In animal models, they are derived from fetal brain homogenates, from which they are cultured as spherical aggregates (neurospheres), which can be in turn be subcultured for \textit{in vitro} differentiation or \textit{in vivo} transplantation. Stem cells for use in humans pose several problems. Their nature as multipotent cells risks unrestrained proliferation to desired or to unwanted cell lineages. They must be derived from fetal tissue in order to preserve the full range of pluripotency, or be obtained in the adult from brain or spinal cord biopsy; the latter as a source may provide cells whose lineage is more restricted to a neuronal phenotype. Cross-species implantation will not avoid immune surveillance. When injected into the cord following contusion injury (Cao, Zhang et al. 2001) or a hemi-section model (Chow, Moul et al. 2000), neurospheres tend to adopt an astrocyte morphology and the neurite connections which do form tend to create pain circuits (Hofstetter, Holmstrom et al. 2005). That indeed no study has shown large scale neuronal differentiation of engrafted stem cells suggests the injured cord, regardless of the injury model, is not a permissive environment (Enzmann, Benton et al. 2006). There have been numerous efforts to control cell lineage with growth factors, location and timing of cell harvest, and genetic transduction prior to implantation.

Many researchers have turned to polymer scaffold substrates as a mechanism to differentiate the cells into neurons either \textit{in vitro} prior to implantation, \textit{in vivo} in conjunction with a scaffold implant, or to encapsulate the cells with polymer for immune protection (Zhong and Bellamkonda 2008). Spinal cord scaffolds then may well play a central role should neural stem cells become useful for human clinical therapy. Recent \textit{in vitro} work has set out to define which polymer compounds and structures are permissive or inhibitory to stem cell
viability and differentiation. The most promising substrates include the use of 3-dimensional fibrin scaffolds, seen in vitro to support and differentiate mouse embryonic stem cells when cell culture and scaffold fabrication conditions were optimized (Willerth, Arendas et al. 2006). Further work evaluated the effects of growth factors, their combinations on stem cell differentiation to neurons and oligodendrocytes (Willerth, Faxel et al. 2007). PLGA has also been shown to support NSC viability and neurite outgrowth to a greater extent than poly-ε-caprolactone (PCL) and PLA (Bhang, Lim et al. 2007). In vivo work done by the Langer lab has used PLGA scaffolds seeded with NSCs in a hemisection model: this scaffold had complex architecture, with an inner layer seeded with NSCs approximating the gray matter, and an outer layer whose pores were oriented longitudinally for axon guidance and radially for permeability. Scaffolds were left in the animal for up to a year with persistent functional improvement, including hindlimb weight bearing and improved coordination seen between 2-3 months post implantation (Teng, Lavik et al. 2002). Our group has also seeded neurospheres into multichannel PLGA scaffolds in a direct comparative study with Schwann cells, Figure 1.9, demonstrating the capacity of PLGA to support neurosphere differentiation (Olson, Rooney et al. 2009).

Micro and nano structures influence stem cell growth and differentiation in vitro. A micropatterned polystyrene surface provided growth differentiation and direction to hippocampal stem cells with laminin (Recknor, Sakaguchi et al. 2006) and with growth factor cues without adhesion molecules (Oh, Recknor et al. 2008). Fibrous nanostructures differentiate NSCs and may even control cell lineage through the structure itself. Silva et al showed self-assembled nanofibres could rapidly differentiate NSCs to neurons and inhibit the development of astrocytes (Silva, Czeisler et al. 2004) Similarly, electrospun nanofibres of PCL polymer differentiated cells and provided a stimulus and directionality to neuron growth (Xie, Willerth et al. 2009).
1.5 Biomaterials as Drug and Cell Based Delivery Systems in the Spinal Cord.

The therapeutic potential of spinal cord scaffolds is enhanced by the development of integrated polymer drug delivery systems and cell lines that are genetically modified to secrete neurotrophic factors. Polymeric delivery from scaffolds is achieved by means of the materials inherent properties: its porosity and permeability for the sequestration and diffusion of a drug its degradation kinetics for release of the entrapped drug, its chemical affinity to a drug by means of a linker moiety, or by being a non-biodegradable material for the preservation of a gradient (Willerth and Sakiyama-Elbert 2007). We have seen in the discussion of individual polymer types a number of ways each polymer type can be used to deliver neurotrophins or drugs. The drug or factor can be released from the material itself, from integrated micro- or nano-spheres or tubules of a different material, or by means of a scaffold’s capacity to support a genetically-modified cell line in vivo.
1.5.1 Micro- and Nano-Spheres and Nanoshells

Micro- and nano-spheres refer to particulate synthetic polymer of the order of microns or nanometers in diameter. By far the most frequently used polymer is PLGA, as its degradation rate and thus drug release is readily controlled by the proportion of PLA to PGA. For example 85:15 PLA to PGA will degrade...
significantly more slowly than a 50:50 ratio. PLGA microspheres are typically 2-45 µm in size. Alginate-chitosan microspheres are also used. Micro and nanospheres are produced by microemulsion techniques, whereby an aqueous solution is emulsified in an organic phase polymer solution to create spherical droplets which are then extracted into another external aqueous phase (Benoit, Faisant et al. 2000). The size of the droplet can be controlled by the emulsion agitation rate, the aqueous and organic phases used, and the addition of surfactants to modify the surface tension between the phases (Khademhosseini and Langer 2007). The drug is usually stabilized with protein (bovine serum albumen) or with zinc, added to the aqueous phase and becomes encapsulated upon polymerization of the droplets. Other techniques include aerosol freeze drying.

The rate of drug delivery will depend on the initial concentration in the sphere and the size of the sphere for a polymer of given degradation kinetics. The particles are injected as a suspension, or can be suspended in a scaffold of another material, in which case the pore size of the material must enable diffusion or cell access within. Alternatively, as shown in chitosan scaffolds (Figure 1.10), the spheres can be localized directly to the channel wall by centrifugation (spin-coating) techniques (Kim, Tator et al. 2008). Nanoshells may be useful in the delivery of hydrophobic drugs from scaffolds. These spherical particles combine the benefits of PEG liposomes with polymeric shell, and are fabricated from PLGA polymer, lecithin phospholipid and a PEG core using modified emulsion techniques (Chan, Zhang et al. 2009).

*Figure 1.10: (A, B) Light micrographs of the microsphere-loaded chitosan channels. The thickness of the channels when hydrated is approximately 200 µm, of which the secondary chitosan layer (indicated by arrows) contributes about 20 µm. (C) Scanning electron microscopy shows microspheres (arrowheads) embedded by the secondary chitosan coating. Figure from (Kim, Tator et al. 2008) with permission.*
Microspheres in scaffolds allow the opportunity for sustained local delivery of therapeutic molecules within the blood-brain barrier. Neurotrophins embedded in PLGA microspheres have been used in a variety of polymer scaffolds with NGF (Mahoney, Krewson et al. 2006) being the most extensively characterized. Cyclic AMP has been shown in many models to induce or enhance axonal growth (Murray and Shewan 2008). Similarly the work of Bunge and colleagues has demonstrated that neurotrophins enhance axonal growth in regenerating spinal cord (Blits, Oudega et al. 2003) and that this may be further enhanced by c-AMP (Pearse, Pereira et al. 2004). Chondroitinase ABC (ch-ABC) an enzyme which degrades a variety of chondroitin and heparin sulfate proteoglycans (Plant, Chirila et al. 1998) as well as hyaluronan, disrupts the glial scar matrix and facilitates axonal repair. Treatment with ch-ABC significantly enhanced axon regeneration in vitro (Plant, Harvey et al. 1995) and following brain (Nash, Borke et al. 2002) or peripheral nerve lesions (Richardson, McGuinness et al. 1980) and, most importantly, SCI in rats (Stichel, Hermanns et al. 1999). Furthermore, functional recovery in rats after SCI has been observed (Bradbury, Moon et al. 2002). We have demonstrated sustained release of dibutyryl cAMP for over 3 weeks when incorporated into microspheres. Finally, while the efficacy of systemic delivery of methyprednisolone in the acute setting for SCI remains an area of contention, polymeric delivery of the steroid is more beneficial in animal models than systemic steroid in reducing the lesion volume when delivered from a scaffold (Kim and Martin 2006) or from an injectible nanoparticle colloid (Kim, Caldwell et al. 2009).

1.6 Conclusions and Future Directions

This chapter has highlighted some of the novel tissue engineering approaches to spinal cord repair using implantable polymers in thoracic transection models. Restoration of respiratory function will be a critical application for biomaterial approaches given the degree of mortality and morbidity associated with respiratory compromise after spinal cord injury. Important bioengineering considerations, fabrication techniques for macro- and micro-engineering, along with some prominent applications and modifications of
the main materials in use for scaffolds, have been presented as an overview of the field. Regarding polymer materials, these continue to evolve rapidly in the sophistication of their chemistry and design. New applications with the polymer Polypyrrole (Ppy) represent an exciting avenue for nanofibre and drug delivery systems. Used in the past as coating for neuroelectrodes, Ppy is electrically conductive, and voltage stimulus can induce axonal growth and its direction, along with the release of integrated neurotrophic factors and dexamethasone from the polymer surface.

The integration of Schwann cells, olfactory ensheathing cells and neural stem cells into the polymer structures have improved their regenerative capacity, as has the use of scaffolds as delivery devices for therapeutic agents, particularly neurotrophic factors. Designing scaffolds and seeded cell lines to release neurotrophins for specific axonal locations and sensitive phenotypes may regionalize repair. The use of human embryonic stem cell implantation in polymer scaffolds will be directed by recent FDA approval for Phase 1 clinical trials, and the outcomes of renewed studies in animals. The absence of their use has the influenced development of alternate stem cell types for scaffolds including mesenchymal stem cells and induced pluripotent stem cells (iPSCs) as potential sources both of new neurons as well as drug delivery cells. Further advances in polymers as genetic delivery tools, particularly for siRNA, non-viral and viral gene delivery with or without targeted genomic integration, is an exciting therapeutic prospect. It is hoped that combination strategies will maximize our ability to regenerate spinal cord tissue through the glial scar and recreate connections. The caveat of course is that the investigation of many possible combinations of material, geometry, functionalization strategies, and integrated drug or cell based therapies will need to be properly controlled and systematic. The ability to control these variables with increasing precision, through rapid technological advances such as were highlighted here, enables scaffold models of spinal cord repair to be highly informative about individualized facets of the repair process.
Chapter 2:
Cell Characterization, Scaffold Fabrication and Implantation in Vivo

2.1 Introduction

Chapter 1 reviewed current tissue engineering and novel therapeutic approaches to spinal cord repair using polymer scaffolds (Madigan, McMahon et al. 2009). The concept of developing 3-dimensional tissue-engineered scaffolds for spinal cord injury has been more extensively explored as a solution for restoring patient function. The spinal cord lacks a support matrix equivalent to the endoneurium and perineurium in peripheral nerve, one that can act as a cellular conduit to approximate disconnected axonal groups. Furthermore, the axonal density of the spinal cord far surpasses that of peripheral nerve with less extracellular matrix support. The rationale for polymer scaffolds is to provide structural support for axonal regeneration, combined with integrated polymeric and cellular delivery systems for therapeutic drugs and for neurotrophic molecules.

Both natural and synthetic biodegradable materials have been used in spinal cord injury repair. Hydrogel polymers (natural and synthetic) are particularly attractive materials for use in the spinal cord. They are macroporous, soft materials readily allowing cell adhesion, migration, and nutrient-waste exchange with the CSF. They can be easily shaped to fit the defect, and their elasticity and degradation may be adjusted by component density. Scaffolds or patterned substrates derived from natural materials such collagen, agarose and alginate, fibrin, fibronectin, chitosan, and laminin have been proposed. Using synthetic polymers offers scope to design and control the characteristics of the material. Early spinal cord scaffolds were made from synthetic polymers that have been used as absorbable sutures for a number of years, poly lactic and poly glycolic acid (PLGA) based materials (Moore, Friedman et al. 2006). Synthetic scaffolds used more recently include biodegradable hydrogels based on
polyethylene glycol (Dadsetan, Szatkowski et al. 2007) or are non-biodegradable hydrogels based on methacrylate (Tsai, Dalton et al. 2006).

Cells may be placed within the scaffold. It has been shown that a variety of cells support axonal regeneration in the cord. These include Schwann cells or peripheral nerve grafts, olfactory ensheathing glia, oligodendrocytes, embryonic sensory neurons (Rosenbluth, Schiff et al. 1997), embryonic stem cells (McDonald, Liu et al. 1999; Chow, Moul et al. 2000; Cao, Benton et al. 2002; Castellanos, Tsoulfas et al. 2002; Hofstetter, Schwarz et al. 2002; Teng, Lavik et al. 2002; Wu, Suzuki et al. 2003) and fetal spinal cord tissue. It is critical for cells to survive in the grafted material (natural or synthetic). While these cell types have been extensively studies in a variety of animal models, few studies directly compare the efficiency of axonal support of cell types in parallel in the same model.

2.1.1 Relevance of the Thesis Project to Ireland and The National University of Ireland, Galway.

Spinal cord injury in Ireland

In Ireland, the incidence of Spinal Cord Injury (SCI) is 13.1 per million population, affecting a young group of people, of median age 37, that is predominately male (78%). There are 1000-1200 people living in Ireland who have sustained this injury. Of the 46 patients admitted to the National Rehabilitation Hospital in Dún Laoghaire in 2000, 23 patients had cervical injuries, 19 had thoracic, and 18 patients in all (40%) had complete loss of function below the level of the lesion (O'Connor and Murray 2006). Road traffic accidents accounted for more than half of the injuries in Ireland, along with traumatic falls, medical causes, causes unknown, sports injuries, and assaults. The average inpatient stay is 9 months, during and following which the patient’s life, in virtually all aspects, is profoundly changed.

A recent assessment of injury burden within 6 European countries including Ireland demonstrated that skull–brain and spinal cord injury resulted in the highest total years lived with disability (YLD) due to lifelong disability in a relatively young male patient group (Polinder, Meerding et al. 2007). Increasingly, injured patients are living near-normal life-spans, given clinical
advancements in immediate and long-term care, particularly respiratory care, along with highly specialized physical rehabilitation, psychological and social service support. In the United States 45.7% of the 253,000 persons living with the residual of spinal cord injury have permanent and complete paraplegia or tetraplegia (National Spinal Cord Injury Database - http://users.erols.com/nscia/resource/factshts/fact02.html). This value of 40% of patients never to experience neurologic recovery holds true in Ireland and in the United States.

**Collaboration between the National University of Ireland, Galway and the Mayo Clinic, Rochester, Minnesota, USA**

A strength of this thesis proposal is to extend international expertise in polymer chemistry, neural cell and mesenchymal stem cell biology, SCI injury animal models, and quantitative assessment of regeneration to advancing therapy of cord injury. An international collaboration has developed over the past 7 years for the advancement of polymer-based therapeutics and translation to patients with spinal cord injury. The Regenerative Medicine Institute (REMedI) and the Network of Excellence for Functional Biomaterials (NFB) within the National University of Ireland in Galway (NUIG) have formed a strong link with investigators at the Mayo Clinic, Rochester, Minnesota, USA. A major strength of the REMEDI team is in the field of adult stem cell biology along with dedicated vector design facilities, the National Gene Vector Laboratory for the production of clinical grade vectors. The group in Galway extends to the Department of Anatomy, and the National Center for Biomedical Engineering Sciences (NCBES). The Mayo Clinic group involves the Department of Neurology and Professor Anthony Windebank’s Neurocellular Biology Laboratory and the Departments of Orthopaedic Surgery and Biomaterials under the leadership of Dr. Michael Yaszemski. There is now a group of over 20 scientists focusing specifically on tissue engineering and gene therapy approaches to spinal cord injury. Over the past 3 years, this collaborative group has produced 29 peer-reviewed publications through directly this collaboration. Two senior investigators in the Mayo Clinic are surgeons, a spine specialist (Yaszemski) and a neurosurgeon (Spinner) with active operative practices who continually inform
decisions about the practicality of applying tissue engineering approaches to patients.

2.1.2 Research at the NCBES and REMEDI, National University of Ireland, Galway

Data for this thesis has been generated in part through a 3 year research project grant awarded by the Health Research Board of Ireland 2007. This grant was co-written by me and Dr. Siobhan McMahon, based largely on NIH RO1 proposals submitted by Professor Windebank. 15 months of my thesis time was spent in the United States, in Professor Windebank’s Neurocellular Biology Laboratory, as a Research Fellow within the Mayo Clinic Department of Neurology. Work at Mayo Clinic was made possible by the National University of Ireland Travelling Studentship in the Sciences award, a national level scholarship promoting international collaboration.

Prior to starting this portion of the project, several key steps had been taken by the REMEDI group and within the Mayo Clinic.

1) (McMahon, Conroy et al. 2006) had reported on the relative efficiency of using adenovirus, adeno-associated virus, lentivirus, and plasmid DNA with lipofection or electroporation to transduce/transfect MSC’s.

2) (Rooney, Moran et al. 2008) had completed a study demonstrating that MSC’s could be transduced to express Nerve Growth Factor by adenoviral gene transfer. I began making constructs for the expression of neurotrophin-3 from lentiviral gene transfer to rat eGFP MSC’s (O'Callaghan, Madigan et al. 2004). We had also shown a lentiviral vector expressing NT3 which I designed, efficiently transduced primary astrocytes and increase Dorsal Root Gangion (DRG) neurite outgrowth in vitro.

3) (Rooney, McMahon et al. 2009) had injected GDNF expressing MSCs into a rat contusion injury model, and a PhD thesis investigating the potential for MSCs to differentiate in neuronal lineages within the spinal cord was published
(Rooney 2007). The work highlighted the survival of MSCs within the cord without evidence that they differentiated into neuron cell types.

4) Lentivirus encoding shRNA (LVsi-RNA) to Neuroglycan-2 had been developed to reduce the expression of NG2 mRNA and protein in Neu7 astrocytes. The Neu7 cell line is an established model for the overexpression glial scar proteoglycans. This work led to the submission of another PhD thesis in REMEDI (Donnelly 2010), and a publication (Donnelly, Strappe et al. 2010) detailing the use of lentiviral shRNA against NG2. The knockdown of NG2 via shRNA targeting and over-expression of NT-3 significantly increase neurite growth in a cell culture model of the glial scar.

2.1.3 Research by the Spinal Cord Repair Group, The Mayo Clinic, Rochester, Minnesota, U.S.A.

At Professor Windebank’s laboratory in the Mayo Clinic, extensive work had led to the development of poly (lactic-co-glycolic) acid (PLGA), poly(ε-caprolactone fumarate) (PCLF) and photocrosslinked oligo-poly((ethylene glycol) fumarate) hydrogel (OPF) scaffold grafts as a platform for studying and optimizing axon growth in the injured cord.

1) (Friedman, Windebank et al. 2002) and (Moore, Jabbari et al. 2004) developed a polymer scaffold approach to spinal cord injury in rats utilizing a transection model, and computed-tomography to study scaffold porosity. The scaffolds were implanted into the rat spinal cord (Moore, Friedman et al. 2006) and supported robust regeneration, representing the first time that complex neural differentiation (myelination) essential for regeneration had been demonstrated on a non-biological substrate. Computer-aided rapid prototyping and vacuum molding was used to construct the graft, which contained seven parallel-aligned channels with internal diameters of 450 μm. Subsequently, in association with REMEDI, it was shown that gene-modified mesenchymal stem cells expressed functionally active nerve growth factor on an engineered poly lactic glycolic acid (PLGA) substrate (Rooney, Moran et al. 2008).
2) (Jabbari, Wang et al. 2005) described the synthesis of PCLF as a novel, injectable polymer for spinal cord repair. Quantified approaches to define the chemical and physical characteristics of scaffolds supporting neural regeneration were published (de Ruiter, Onyeneho et al. 2008). These included stiffness, degradation, swelling, permeability, ability to hold a suture and surface characteristics. Subsequent studies (Wang, Yaszemski et al. 2009) demonstrated that ‘surface etching’ PCLF with alcohol improved Schwann cell adhesion and neurite outgrowth.

3) Reliable methods of axonal tracing were developed by (Chen, Miller et al. 2006), demonstrating that axons regenerate from the spinal cord in both directions through a PLGA polymer scaffold. Axon tracing with Fast Blue defines the origin and path of axons. Regenerating axons penetrated several millimeters into the cord after re-entry from the scaffold.

4) Animal models for spinal cord injury were discussed and evaluated in light of tissue engineering strategies for neuronal regeneration (Talac, Friedman et al. 2004).

5) (Krych, Rooney et al. 2009) began to demonstrate the effect of macroarchitecture on axonal regeneration through PLGA scaffolds loaded with Schwann cells. The study directly compared PLGA scaffolds with two different channel size, either 450 mM (n=19 animals) or 660 mM diameters (n=14 animals). Larger channels had diminished capacity to support regeneration and a much higher proportion of peripheral fibrosis was noted around the channel wall.

6) (Olson, Rooney et al. 2009) directly compared the capacity of Schwann cells and neural stem cells (NSC) to support axonal regeneration in the spinal cord through PLGA scaffolds. The best results were seen with Schwann cells with total axonal counts approaching 1000 axons per scaffold.

7) (Dadsetan, Szatkowski et al. 2007) described the synthesis of a novel hydrogel polymer (OPF). Its initial applications in bone repair were quickly extended to use with bone marrow derived MSCs and their differentiation
potential on this substrate (Dadsetan, Hefferan et al. 2008). Chemical modification to include a positive charge within the hydrogel significantly enhanced neuronal cell attachment, Schwann cell migration and axonal myelination *in vitro* (Dadsetan, Knight et al. 2009), making this an attractive candidate to investigate further as scaffold implant material *in vivo*.

8) Finally, (Chen, Knight et al. 2011) have completed a direct comparison of PLGA, PCLF, neutral OPF and positively charged OPF scaffolds each loaded with the best cell type to date, Schwann cells. The study has concluded that OPF+ scaffold are superior in supporting regeneration to the other polymers, and that the architecture of the regenerated tissue is more orderly in its structure than that seen in the other polymers. A high density of axons were confined to the scaffold channel core in the OPF+ group.

2.1.4 Rationale for the Current Project Aims

The use of MSCs in spinal cord regeneration has gone beyond the hope that they will differentiate into neural phenotypes *in vivo*. An investigation of MSCs’ own capacity to support axonal regeneration, and what role they do play when introduced into the central nervous system following spinal cord transection injury, is a key aim of the thesis. The focus is also shifting now towards use of MSCs for growth factor delivery following their genetic modification with virus.

In this project, the polymer scaffolds themselves have evolved from structurally rigid polymers made of PLGA to softer polymers such as PCLF and OPF hydrogels. We are now investigating how the regeneration microenvironment can be adapted with modifications to polymer surface charge. In a direct comparison, positively charged OPF hydrogel is the optimal polymer material studied in the laboratory to date in supporting axonal regeneration, and thus it was selected in this project as the scaffold polymer of choice.

Schwann cells were more efficient than neural stem cells in PLGA scaffolds, and thus are the most efficient cell type to date in supporting axon regeneration. Schwann cells were chosen for direct comparison with MSCs in OPF+ scaffolds implanted in a transection model.
Chapter 2 will describe the isolation of rat eGFP-MSCs and their characterization as stem cells capable of phenotypic differentiation to mesenchymal lineages. The isolation and characterization of Schwann cells from neonatal rat pups is also described. OPF polymer synthesis, scaffold fabrication, scaffold cell loading with MSCs and Schwann cells, spinal cord transection surgery and scaffold implantation in rats, post operative care and functional analysis, tissue harvesting and histology processing are detailed in the following sections.
2.2 Materials and Methods

2.2.1 Cell Culture

*General procedures*

All cell culture was performed in a class II biosafety laminar flow hood and under aseptic conditions. Hoods were either open laminar flow or shielded flow hoods. Prior to all cell culture work, the hoods were sterilized with Virkon S disinfectant and virucide (active ingredients 21% potassium peroxymonosulfate and 9.75% available chlorine (Dupont Chemical) and then with 80% ethanol. Cell media and trypsin was pre-warmed to 37°C in water baths containing autoclaved water with SigmaClean water treatment (800 ul per 5 litres). Cells lines were cultured in growth medium as detailed under the specific cell type, below. For passaging of cell lines in T75 flask, 30 mls of growth medium was removed from cells and cells were rinsed gently with 5 ml sterile 1 X PBS. The PBS was removed and 3 ml prewarmed 1 X trypsin was added to the flask, gently rotating the surface to ensure that the trypsin sufficiently coated the cells. The cells were returned to the cell culture incubator for 3-5 mins. The cells were quickly visualized at 10x power under microscopy, and when sufficiently detached, the trypsin was neutralized by adding 7 mls of prewarmed growth medium containing 10% FBS. The cells were then triturated to yield a single cell suspension, which was then centrifuged at 500g for 5 minutes. The cell pellet was resuspended in 5 ml media.

Cells were counted using a haemocytometer. A 50 ul aliquot was diluted in 150 ul cell media for a 1:4 dilution, 10 ul of which was loaded onto each side of the cytometer. The cells were counted in the central box in duplicate, and the number per ml was calculated by multiplying the average counted number by the factor 10,000 and by the dilution factor of 4. Typically cells were split at a 1:4 to 1:8 ratio, depending on the original count. The appropriate volume of cell suspension was added to a T75 flask containing 30 mls of prewarmed growth medium, and returned to the cell culture incubator for culture.
For freezing cell lines, 100,000 cells per vial were aliquoted into 15 ml conical tubes, centrifuged at 500g for 5 mins, re-pelleted in undiluted FBS with 10% DMSO and transferred to cryovials. The cells were frozen in storage containers with surrounding baths of isopropyl alcohol. The containers were initially at room temperature, and subsequently transferred to the -80 °C freezer for graded temperature decreases as per their design. The vials were transferred to liquid nitrogen within 48 hours.

To use the cells from storage, vials were removed from liquid nitrogen, placed on ice until use, and then dipped directly into a 37°C water bath for rapid thawing. Once thawed the cells were added to the bottom of a 50 ml conical tube, and 10 ml pre-warmed tissue culture media was added drop wise slowly. This procedure was thought to limit osmotic stress on the cells, and rinsed them clear of residual DMSO. The cells were pelleted at 500g for 5 mins, and resuspended in 30 ml of culture media for transfer to T75 flasks. After 24 hrs the cells were fed by replacing the media fresh prewarmed growth medium, and the cells were cultured as appropriate to their use.

### 2.2.2 Rat MSC Isolation and Characterization

**Surgical Procedures**

Rat MSC (MSC) primary cultures were obtained from femur and tibia bone marrow, as we have previously described (Rooney, Moran et al. 2008). After euthanasia, hind limb long bones were surgically isolated from wild type (wt) and enhanced Green Fluorescent Protein (eGFP)-transgenic Sprague Dawley (SD) animals (CZ-004 [SD TgN(act-EGFP) OsbCZ-004], Genome Information Research Center, Osaka University, Japan, n=18). The rats were pinned in on their backs with an 18G needle through the loose skin around the axilla and through the foot of each hind limb. An incision was made to expose the lower limbs, the muscle mass was cleaned with blunt dissection, and the limb was excised at the hip joint. The bones were again cleaned of muscle and cartilage and separated at the knee joint. The fibula and foot were removed using a bone cutter. The femoral and tibial bones were transferred initially to ethanol to enhance sterility and then into sterile Tyrode’s solution on ice. The proximal end of the femur and the distal
end of the tibia were removed using sterile bone cutters and the shafts were placed in complete medium.

**MSC isolation**

Bone marrow was flushed into 100mm cell culture dishes containing 25 ml complete MSC media (50:50 mixture of α-Minimal Essential Media (α-MEM) to Ham’s F12 culture media, 10% Fetal Bovine Serum, 1% antibiotic-antimycotic (Gibco-Invitrogen, Carlsbad CA)) by inserting a 21G needle through an intact proximal epiphysis and injecting α-MEM distally through the open, transected end. Marrow plugs were dissociated by pipetting, and centrifuged at 500g for 5 mins. All but ~ 0.5cm of the cleared supernatant was removed using a 25ml serological pipette leaving the soft pellet, to which another 25 ml of media was added. The suspensions were transferred through each of the tubes and the pellets were pooled to a final volume of 30mls of media. A 50μl aliquot of the cell suspension was mixed with 450μl of D-PBS. 50μl of this solution was added to 50μl of 4% acetic acid for 1min to lyse red blood cells, and the cells were counted using a hemacytometer by placing 10μl of the mixed solution on either side of the cover slip. Cell suspension was passed through a 70 μm filter, plated at a density of 9 x10^5 cells/cm² (166 x 10^6 cells per T175 flask) and cultured at 37ºC in 5% CO₂/90% humidity. MSC cultures were obtained from the marrow suspension by differential adherence to the growth substrate, removing non-adherent cells after 3 days. Cell colonies grew to suitable density for subculture after 16-17 days, and were dispersed at 5.7 x 10^3 cells/cm². Cells were maintained in complete MSC media and used experimentally up to passage 4.

**Flow cytometry analysis**

eGFP-MSCs were trypsinized, distributed into aliquots of 3 x 10^5 cells in 1.5 ml Eppendorf tubes. The cells were pelleted in a microfuge (3000-5000 rpm x 5 minutes) and fixed in phosphate buffered saline (PBS) with 4% paraformaldehyde for 20 minutes. The cells were pelleted and rinsed once in 500 ul PBS and re-pelleted. Cells were then incubated with primary antibody diluted in 400 ul PBS with 5% normal goat serum for 1 hour. Primary antibodies to CD90 (Thy-1.1) (mouse anti-rat 1:200), CD11b/c (macrophage) (mouse anti-rat, 1:200) (Biolegend, San Diego, CA), CD73 (ecto 5’ nucleosidase) (mouse anti-rat, 1:100),
CD45 (pan-leukocyte) (mouse anti-rat, 1:200), CD71 (Ox-26) (mouse anti-rat, 1:100), and CD172 (Ox-41, Signal Regulatory Protein (SIRP)) (mouse anti-rat, 1:100) (BD Biosciences, San Jose CA) were used. Following incubation with the primary antibody, the cells were again pelleted and rinsed in 500 ul PBS + 5% goat serum, following which a goat-anti mouse IgG (H+L) secondary antibody conjugated to AlexaFluor® 647–R-phycoerythin (PE) (Invitrogen) was used at a dilution of 1:100 in 400 ul PBS with 5 % goat serum for 1 hour. The cells were pelleted and rinsed in PBS and resuspended in 400 ul and transferred to 10 ml polystyrene FACs tubes. Testing for CD105 (endoglin) positive marker and CD34 (haematopoetic progenitor) negative marker was not possible given that suitable antibodies against rat antigen were unavailable. FACS analysis for eGFP and PE signal was performed on 50,000 events (FACS Calibur apparatus, Becton-Dickinson, Franklin Lakes NJ), with suitable controls including cells only, cells with secondary antibody only, isotype control, and positive and negative fluorescent controls. The analysis for CD90, CD73, CD45 and CD 11/b/c antibody labelling was done by me and ran through the FACS apparatus by technicians in the Mayo Clinic Flow Cytometry and Cell Sorting core facility. The analysis for CD71, CD172, and the isotype control were done by Dr. Gemma Rooney within REMEDI, analysed using the Guava cytosoft machine, using the ExpressPlus software (Guava Technologies, Philadelphia, USA). The work is referenced in her September 2007 PhD thesis for the National University of Ireland, Galway (Rooney 2007). Antibodies used for FACS analysis are listed as Table 2.1, in the appendix to Chapter 2.

**Wild type MSC and eGFP-MSC differentiation assays**

The capacity for MSC cultures to differentiate into adipocyte, osteocyte, and chondrocyte cell lineages was assessed as we have previously described (Rooney, Moran et al. 2008). This analysis was done on the initial batch of cells by Dr. Gemma Rooney, Department of Neurology, Mayo Clinic, but not on subsequent passages (Rooney 2007; Rooney, Moran et al. 2008). eGFP-MSC monolayers of 250,000 cells per well in a 6-well plate were cultured in adipogenic induction media (high glucose DMEM with 10% FBS, 1% antibiotic-antimycotic (Gibco), 5% rabbit serum, 1 µM dexamethasone, 200 µM indomethacin, and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis MO)) for 3 days. At this
time cultures were switched to adipogenic maintenance media (high glucose DMEM with 10% FBS, 1% antibiotic-antimycotic (Gibco), 5% rabbit serum, 10µg/ml insulin) for 1 day before replacing the media again with induction media. After 3 induction-maintenance cycles, cells were fixed with 10% neutral buffered formalin and lipid vesicles were stained with Oil Red O (Sigma-Aldrich) for light microscopy and absorbance measurement of extracted Oil Red O at 495 nm.

eGFP-MSC monolayers of 30,000 cells per well of a 6-well plate were cultured in osteogenic media (α-MEM with 10% FBS, 1% antibiotic-antimycotic (Gibco), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 µM ascorbic acid 2-phosphate (AA2-P) Sigma-Aldrich), changing cells media every 3 days. At 17-20 days of culture, cells were visualized with light microscopy, and measurement of calcium mineral deposition was performed as previously described (Jaiswal, Haynesworth et al. 1997).

Chondrogenesis was evaluated over a three week course of pelleted cell culture at 37ºC. 5 x 10^5 cells (n=4 per group) were centrifuged at 800g for 5 mins and maintained in chondrogenic media containing high glucose Dulbecco’s Modified Essential Media (DMEM) with 10% FBS, 1% Antibiotic-Antimycotic, 1 mM sodium pyruvate (Gibco-Invitrogen), ITS + Supplement (6.25 µg/ml bovine insulin, 6.25 µg/ml bovine transferrin, 6.25 µg/ml bovine selenous acid, 5.33 µg/ml linoleic acid, 1.25mg/ml BSA, 100nM dexamethasone, 50 µg/ml AA2-P, 40 µg/ml proline (Sigma-Aldrich), 10 ng/ml transforming growth factor-β3 (TGF-β3) and 100 ng/ml bone morphometric protein-2 (BMP-2) (R&D Systems, Minneapolis MN). Media was changed every 2 days. At intervals of 14 and 21 days, pelleted cultures were assayed for their concentration of chondroitin-6-sulfate by reading the absorbance at 595 nm of 1,9 dimethylmethylene blue (DMMB) in dilution buffer (50 mM sodium phosphate, pH 6.5, 2 mM N-acetyl cysteine, 2 mM EDTA), against a standard curve of range 0-10 µg/ml (Sigma-Aldrich) as described (Petit, Masuda et al. 1996).
2.2.3 Rat Schwann cell Isolation and Characterization.

**Surgical procedures**

Rat Schwann cells (SCs) were cultured from the sciatic nerves of two to five day old newborn pups (n=15-18), as we have described (Olson, Rooney et al. 2009). Litters of pups were separated from their parent, and each injected with 0.1 ml SleepAway Solution (26% sodium pentobarbital, 7.8% isopropyl alcohol, 20.7% propylene glycol in distilled water), injecting directly into the liver or abdomen. The pup was placed face down and sprayed with 80% ethanol. Sciatic nerves in both limbs were surgically removed. The skin above the base of the tail was opened with scissors, an inverted Y-shaped cut was centred up the spine and down the posterior skin of both legs and the skin was pulled out of the way. The sciatic nerves were visualized under a dissecting scope (Zeiss Stemi 2000-CS instrument) by bluntly separating the hamstring muscles from their plane with the quadriceps along the leg. When clearly visualized along their length, the nerves were cut with spring-loaded dissecting scissors proximately at their spinal origin and distally in the popliteal fossa. The nerve segments were placed in petri dishes containing Leibovitz’s L-15 culture media (Gibco).

**Schwann cell isolation**

The nerves were transferred to the sterile flow hood and were stripped of their fibrous perineurium using fine forceps under a dissecting scope. The perineurium could often be removed in strips, or as a single tubular sheath that came away from the nerve by inverting upon itself. The remaining nerve fascicles were minced and digested in 2.5 mg/ml trypsin and 0.3 mg/ml collagenase (Sigma-Aldrich) in Hanks Balanced Salt Solution (HBSS) (Gibco) for 45 minutes at 37°C. Complete SC media (50:50 DMEM to Ham’s F12 media, 10% FBS, 1% penicillin-streptomycin (Gibco)) was added to stop the reaction, and the digest mix was mechanically dissociated by pipetting 20-30 times with a glass Pasteur pipette. The mix was transferred to a 50 ml conical tube, and centrifuged at 500g for 5 minutes to pellet the heavier nerve tissue debris. The cell suspension remaining in the supernatant was re-plated on laminin coated 35 mm dishes and cultured for 48 hours at 37°C prior to scaffold loading.
SCs were characterized in vitro by immunocytochemistry using antibodies against S-100 (mouse anti-human, 1:300 (BioGenex)) and CD90 (Thy-1.1) (mouse anti-rat 1:500 (Biolegend) for approximation of the proportion of contaminating fibroblasts. Cells were seeded at 20,000 cells per 6-well plate well, fixed in PBS with 4% paraformaldehyde, and counted in 3 random fields at 20x magnification over 3 separate dispersions. SCs in this portion of the project were used as primary cell lines and not serially passaged. Preparations with high proportions of fibroblasts (>20%) were discarded. It was subsequently determined that poor technique in removing the perineurial sheaths resulted in higher proportions of contaminating cells. Image acquisition was performed using a Zeiss AxioCam loaded on a Zeiss Axio Imager Z1 microscope with Zeiss KS400 software (Oberkochen, Germany).

2.2.4 Oligo (poly(ethylene glycol) fumarate) (OPF) Synthesis

OPF with a number average molecular weight (Mn) of 16,246 +/- 3710 was synthesized from poly(ethylene glycol) (PEG) with an Mn of 10,000, according to a previously described method (Jo, Engel et al. 2000).

![OPF Synthesis](image)

**Figure 2.1:** OPF Synthesis. OPF is made by a condensation reaction between polyethylene glycol 10,000 and distilled fumaryl chloride in the presence of triethyamine. The biproducts of the reaction are small organic acids like fumaric acid, from which the OPF is further purified by evaporation, and recrystallization from ethylene acetate.
For purification, methylene chloride was removed by rotary evaporation. The resulting OPF was dissolved in ethyl acetate and filtered to remove the salt from the synthesis. The synthesis was carried out by Dr. Mahrokh Dadsetan in the Mayo Clinic Department of Orthopaedic Surgery and Biomedical Engineering. 50 g PEG was azeotropically distilled in toluene to remove residual water and then dissolved in 500 mL distilled methylene chloride. The resulting PEG was placed in an ice bath and purged with nitrogen for 10 min. Then, 0.9 mol triethylamine (TEA; Aldrich, Milwaukee, WI) per mol of PEG and 1.8 mol distilled fumaryl chloride (Acros, Pittsburgh, PA) per mol of PEG were added in a dropwise fashion. The reaction vessel was removed from the ice bath and stirred at room temperature for 48 hours the reaction of TEA and chloride. OPF was recrystallized in ethyl acetate and vacuum dried overnight to form a cream-coloured powder-aggregate.

2.2.5 Fabrication of OPF+ Hydrogel Scaffolds

Positively charged hydrogels (OPF+) were made by dissolving 1 g OPF macromer in deionized water containing 0.05% (w/w) of a photoinitiator (Irgacure 2959, Ciba-Specialty Chemicals) and 0.3 g of the cross-linking reagent N-vinyl pyrrolidinone (NVP). The OPF hydrogel was chemically modified at 20% w/w with the positively charged monomer [2-(methacryloyloxy) ethyl]trimethylammonium chloride (MAETAC) (80% wt in water, Sigma-Aldrich) (Dadsetan, Knight et al. 2009). MAETAC is a bifunctional molecule containing both a pH-independent cationic head (quaternary ammonium) and a reactive methacroyl group that copolymerizes with the fumarate group of the OPF. The proportions of each chemical component of the hydrogel are listed in Table 2.2, Chapter 2 appendix.
Axonal Regeneration Supported by OPF+ Cell-loaded Hydrogel Scaffolds

Figure 2.2: Oligo (poly(ethylene glycol) fumarate) (OPF) is a PEG based hydrogel incorporating a fumarate moiety. It is made positively charged with the addition of MAETAC to the polymer mix (20% w/w). NVP crosslinks the OPF and MAETAC at the sites indicated with the blue boxes, when reacted with photoinitiator (Irgacure 2959) and UV light at 365 nm (blacklight wavelength). MAETAC is a positively charged quaternary amine.

Figure 2.3: OPF crosslinking. The hydrogel is formed from the liquid polymer mix as a crosslinked network as detailed below, although this depiction does not include the MAETAC moiety.
Hydrogel scaffolds were fabricated by mold injection of liquid polymer cast over 7 parallel wires of 290 µm diameter, aligned within a glass tube of 1.55 mm inner diameter. Teflon plugs depicted here against the backdrop of a millimetre ruler were manufactured with precision drilling by the Mayo Clinic Bioengineering Core to secure the wires in a 6+1 orientation. A wire also depicted laying vertically against the ruler for sizing (Figure 2.4, left) The plugs were carefully threaded with the wires to ensure that the correct alignment was maintained along the length of the tube and to ensure that the wires had not crossed at any point. The liquid polymer recipe was combined in a 50 ml conical tube, which was placed into a 37ºC water bath to improve the rate at which the OPF powder would dissolve. Once dissolved, the tube was centrifuged at 500g for 10 minutes to force out any entrapped air. The liquid mixture was then carefully drawn up into a 1 ml syringe and a 30 gauge needle was attached. The needle was inserted at one end of the mold, forcing it between the wires, and the polymer was injected to fill the mold prior to sliding the proximal plug back into place. Figure 2.5 (above) shows a mold filled with the translucent polymer. The OPF/MAETAC mixture was then polymerized by exposure to UV light (365 nm) at an intensity of 8 mW/cm² (Black-Ray Model 100AP) for 30 min (15 minutes per side). After being left to dry for overnight at room temperature, the polymer would retract away from the glass wall. It was then possible to slide
the glass tube clear leaving an intact column of dehydrated polymer scaffold of 20 mm length overlying the wires. The columns were soaked in sterile PBS water for 1 hour, reaching a final diameter of 3 mm. The wires can be readily removed when the column is fully hydrated. The column was cut into 2 mm lengths for use in animals using a Teflon jig.

![Teflon jig](image)

**Figure 2.6:** A Teflon jig was made through the Biomaterial Engineering Core facility at Mayo Clinic. The device has deep cylindrical grooves to accommodate scaffolds of two diameter types. Perpendicular to these grooves are 2 parallel cuts precisely two mm apart that hold a series of razor blades. Scaffold material is trimmed square at one end and then advanced to abut one razor, while a second makes a cut behind it, leaving individual scaffolds of two mm lengths.

In order to calculate the volume of fluid a 2mm scaffolds contained, the weight of single scaffold pieces soaked in water to their maximum size was measured on a fine balance (Mettler Toledo AG 104). Afterwards the scaffolds were vacuum dried for 24 hours. Again, the scaffolds were weighed and the average difference was used for calculating the amount of water lost during the drying process.

### 2.2.6 OPF+ Scaffold Preparation and Cell Loading

Two mm lengths of OPF+ scaffold were sterilized prior to cell loading by immersion in serial dilutions of ethanol (80%, 40%, and 20% for 30 minutes each), soaked in distilled autoclaved water for an additional 30 minutes and then equilibrated in the appropriate complete cell culture media (MSC or SC media).
for 1 hour. The hydrogel takes up the phenol red and this gives the scaffold a pink colour. MSC or SC cultures were trypsinized, counted and resuspended in undiluted Matrigel™ (BD Biosciences) at a density of 50,000 cells/µL. Matrigel is a proprietary basement membrane composite derived from an Engelbreth-Holm-Swarm (EHS) mouse sarcoma line, which is liquid at 4°C and solidifies into a gel at 37°C. To prepare the Matrigel cell solution, estimation was made that each scaffold would require 8 µl of cell suspension in total, and that the number of scaffolds prepared should be 2 more than the number of animals planned for surgery. Thus, for an n=8 of animals, 10 scaffolds and a total of 80 µl of Matrigel-cell solution was needed, some 4 million cells per group. This typically represented at least 4 confluent T150 flasks of cells per experimental group.

Available flasks were trypsinized, the cell number determined and 4 million cells were transferred to a 50 ml conical tube which was centrifuged to pellet the cells. Undiluted Matrigel in 100 µl aliquots was thawed on ice and then microfuged to remove any air. Care needed to be taken to ensure all the cell culture media was removed from the cell pellet, otherwise the suspension would be diluted with media and would not set properly at 37°C. The appropriate volume of Matrigel was then applied to the cell pellet and this was carefully mixed by pipetting being sure not to introduce any air into the suspension.

Cells were loaded into the scaffold channels using a P20 pipette and tip. The tips had been kept at -20°C overnight, and on ice during the cell loading. The scaffolds were held beneath a dissecting scope with the aid of fine forceps that had a small rubber ring at their apex. When the ring was slid into the correct position, a scaffold could be picked up by opening the forceps between their arms, placing the forceps at the outside edge of the scaffold and allowing the rubber to close the forceps at the correct tension. All seven 450 µm diameter channels were loaded at once by sequentially applying 4 µl of cell suspension to each end of the scaffold’s open face, and drawing the suspension through by touching the opposite end against sterilized tissue wipes. The wipes were sterilized by wrapping them around the outside of a 100 mm petri dish, saturating them with 80% ethanol and then allowing them to dry in the tissue culture hood. When the suspension was applied and drawn through both ends, the scaffold was inspected for any air bubbles forming within a channel, and where possible, the air was
pried to the surface with a 30 gauge needle. The scaffold was then placed into the tissue culture incubator for 2-3 minutes, laying on its edge for the gel to set.

When the suspension was too dilute, the cells within would aggregate in a gravity dependent manner within the channel, which was not optimal for use in the animal. When the suspension had set, the scaffold faces were ‘capped’ with 2 – 3 ul undiluted matrigel or cell suspension (if extra was available). This was done by applying a bleb to the surface of a 60 mm dish, laying the scaffold face down onto the drop, and then applying a second seal to the upward face. The scaffold was then allowed to incubate for another 2-3 minutes to solidify the caps, after which the dish was filled with culture media.

For control animals, scaffolds were loaded with Matrigel alone. The loaded scaffolds were cultured in complete media for 24 hours to use for implantation. Prior to use, the scaffolds were carefully scraped clear of the dish surface by using a number 11 scalpel blade to cut around its circumference and pry it free. Care needed to be taken not to allow the channel contents to be pulled out of the scaffold by the underlying Matrigel seal. The prepared scaffolds were then transferred to individual 60 mm dishes and laid on small pieces of gauze saturated with media for ease of use during the rat surgeries. Approximately 0.68 µL of cell suspension was required to fill each channel, giving a total of 34,000 cells per channel and 238,000 cells per scaffold.

### 2.2.7 Spinal Cord Transection and Scaffold Implantation

**Surgical procedures**

All surgical and care procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic, Rochester MN, with a full review of proposed methodology and requested animal numbers. It was an institutional requirement that formal proposals for animal surgery be written and submitted by me. In addition, all experiments were in accordance with the National Institute of Health and the U.S. Department of Agriculture Animal Welfare Act. Specifically, The Institute for Laboratory Animal Research (ILAR) within the National Academy of Sciences has issued a new edition of the Guide for the Care and Use of Laboratory Animals which was
incorporated into the Mayo Clinic’s IACUC regulations (National Academy of Sciences 2010).

All experiments involved the use of female rats, given the ease of bladder expression and reduced incidence of urinary tract infections compared to males. Female Sprague Dawley rats weighing 230-250 grams (Harlan Laboratories, Madison WI) were anesthetized by intraperitoneal injection of a Ketamine/xylazine mixture, 80 mg/kg and 5 mg/kg respectively (Fort Dodge Animal Health, Fort Dodge, IA). Animals had received acetaminophen (300 mg/kg) in their drinking water for the previous 24 hours. Animal weight was recorded. When anaesthetized, the animal's eyes protected with gel (Puralube Vet Ointment, Pharmaderm, Melville, New York), and each received 0.015 mg buprenorphine (Buprinex, Reckitt Benckiser Pharmaceuticals Inc, Richmond, VA) IM (0.05mg/kg) for pain, 1.5 mg enrofloxacin (Baytril) IP (Bayer Corporation, Shawnee Mission, KS) as a perioperative antibiotic, and 5 ml warmed normal saline (Baxter Healthcare Corporation, Deerfield IL) subcutaneously for fluid maintenance. Hair over the site of incision was clipped, and the surgical field was disinfected using Povidone-Iodine scrub swabsticks (Professional Disposables Inc, Orangesburg, NY). Heating pad temperature beneath the animals was constantly maintained at 37°C during surgery and recovery, either by means of a water bath irrigating a pad, or by electric blankets. Animals were positioned with their forearms draped over a 15 ml conical tube to ensure the correct spinal angle for surgery. Each animal received oxygen and low dose isoflurane during the 15 minute procedure.

Operations were performed using sterile technique and with the aid of a Zeiss Superlux 40 (F-170) microsurgical microscope (Oberkochen, Germany). A 0.75-1 inch incision was made on the dorsal aspect of spine from T7-T10. After blunt dissecting and retracting the subcutaneous fat and facia, the paraspinal musculature was moved laterally away from the dorsal spinus processes, scraping the spinal and transverse processes clear. Laminectomy was then performed through the T8-T10 level using rongeurs directly clipping away the bone arches. The spinal cord was exposed and transected using No. 11 scalpel blade. Complete transection was ensured using a hooked probe. All visible nerve roots within the area of the transection were removed. The bleeding from the spinal cord dorsal
and ventral arteries was sometime quite profuse, and was controlled with gauze and sterile Q-tip cotton buds, and the wound was irrigated regularly with saline.

Once the bleeding had stopped, control (Matrigel only) and cell-loaded OPF+ scaffolds were implanted between the free ends of the spinal cord verifying that there was good contact and alignment with the rostral and caudal stumps. It was not necessary to remove a section of the cord, as when the transection was complete, the cord ends sprang apart under their own tension to an adequate distance to accommodate the scaffold. The wound was closed in layers with absorbable 4.0 vicryl sutures, with the closed muscle and facia layer securing the scaffold in place. It was not necessary to stabilize the spine any further. The subcutaneous fat was closed separately below the skin, which was itself closed with either a continuous or separate suture technique. All animals were monitored regularly until fully conscious, including monitoring of body temperature at three intervals. When they regained consciousness, they were kept warm on heating pads and with sterile surgical towels in their cages for the next 24 hours. Late into the evening of the surgery, animals were inspected again, bladders were expressed, and each received an additional dose of Buprinex and 5 ml saline. After spinal cord transection all the animals were paraplegic and without urinary function.

**Postoperative care**

Animals were housed in individual cages for the first week of recovery and then in pairs, in low walled cages for easier access to food and water. All animals were housed in conventional housing with a standard 12 hour light dark cycle and food and water ad libitum. There was 24 hour access to veterinary care with staff experienced in managing spinal cord injury models. For animals which were unable to feed well, meat dog food or gel based nutrition was provided on the cage floor in addition to normal rat chow. Postoperatively, animals were given enrofloxacin 5.0mg/kg IP twice daily, buprenorphine 0.05mg/kg twice daily IM and 5ml saline solution administered subcutaneously for 5 days. Acetaminophen diluted to 300 mg/kg in the animal’s drinking water was continued for 7 days after surgery.
The anticipated post-operative complications included:

- Hind limb paralysis
- Urinary retention and urinary tract infection
- Lethary and poor feeding
- Decubital ulcers from immobility
- Infection at the wound site.
- Pain behaviour including self mutilation
- Animals in pain were listless with black rings around the eyes and nose.
- Bleeding from the urinary tract without signs of infection.

All animals received twice daily monitoring for wound sepsis and autotomy, with bathing for wound and skin care and topical antibiotics or aluminium spray applied as required. With twice daily bladder expression, the volume and cloudiness or blood in the urine was monitored on a scale of 0 to 4+ in volume. Urinary tract infections were judged on the basis of blood and urine cloudiness, and treated with enrofloxacin and normal saline twice daily for 3 days. Animals were also scored for the degree of autophagia behaviour displayed using a novel scoring system (0 points for none observed, 1 point for mild hair pulling or skin exposure, 2 points for biting with fresh blood or skin puncture, 3 points for open wounds and tissue loss requiring sutures, and 4 points for severe wounds requiring that the animal be euthanized). Cone-shaped collars were applied if there were signs of self mutilation.

Animals who remained moribund longer than anticipated anesthesia recovery time (24 hours postoperatively), animals who were persistently unable to access food and water, and animals who suffered severe skin ulceration or demonstrated severe autophagia were euthanized and removed from the study for humane purposes.

**Basso, Beattie and Bresnahan (BBB) functional analysis.**

Hindlimb locomotor function in experimental and control rats was assessed in open field testing by three independent observers blinded to the treatment at intervals of two and four weeks after surgery. Each rat was observed in an open plastic box for five minutes, and observers recorded individual hind
limb ankle, knee and hip movements, along with weight support, toe clearance, tail position, and the coordination of gait and paw position. Function was given a score on a 21 point scale according to the established BBB locomotor rating scale (Basso, Beattie et al. 1995) (Table 2.3, Chapter 2 appendix). The score is calculated by using the highest movement scores of the two legs observed. Animals were assessed at 2 weeks and then at 4 weeks immediately prior to tissue harvesting.

2.2.8 Tissue Harvesting

Animals were euthanized using methods consistent with the American Veterinary Medical Association. Animals were deeply anesthetized with an IP injection of 0.35ml SleepAway™ sodium pentobarbital solution (26% pentobarbital, 7.8% isopropyl alcohol, 20.7% glycerol) (Fort Dodge Animal Health, IA) followed by transcardial perfusion with 4% paraformaldehyde in Phosphate Buffered Saline. Tissue fixative solution was made up fresh in batches of 1 litre by heating distilled water to 56°C, dissolving 40 g of paraformaldehyde with the addition of 1-2 pellets of solid NaOH for pH adjustment, and then adding the preformed PBS tablets, 5 tablets per litre. The solution was cooled to 4 degrees overnight. Once the animal became unresponsive to pain, it was pinned on its back through the forearm and hindlimbs, and the sternum of was opened with scissors. The heart was isolated with forceps and its apex was pierced using a sterile #11 scalpel blade. A wide bore cannula was inserted through the left ventricle into the ascending aorta and the ventricle wall was sealed tight around the cannula pinching it closed with forceps. After washing with a minimum of 60 mL of phosphate buffered saline (PBS), 60mL of perfusion fixative was injected to flow through the circulation until stiffness of the limbs and tail was observed. The entire vertebral column from cervical to sacral segments was removed, placed in perfusion fixative solution and post-fixed at 4°C for 1 week.

To recover the scaffold, the vertebral column was oriented in space by recognizing the cauda equinal regions and noting the area of rostral scoliosis around the scaffold placement. The tissue was prepped by stripping the paravertebral muscles from the dorsum of the spine, at which point the gap in the spinal processes following laminectomy could be readily felt over the insertion site. Using scissors
the column was trimmed to 1 cm either side of the insertion site, following which rongeurs were used to carefully clip away the vertebrae without damaging the scaffold and spinal cord. When within a few millimetres of the scaffold, the cord plus its implant were cleared of any remaining bone under a dissecting scope and dissected free. An angled cut was made across the cord at its rostral end, and a straight transverse cut across the caudal end. This allowed for orienting the cord and scaffold for when it was embedded in paraffin. The scaffold and cord was stored at 4°C for 7 days in fresh fixative solution.

**Tissue processing for immunohistochemistry**

The spinal cord, cut at an angle at its rostral end, and containing the scaffold, was embedded in paraffin. Embedding and sectioning was done by LouAnn Gross of the Department of Orthopedic Surgery at Mayo Clinic. One day prior to processing, the paraffin was melted, (with dry pellet paraffin yielding about one third the volume in liquid) in a heating pot, and several paraffin/xylol solutions were prepared at respective ratios of 1:2; 1:1; 2:1. Stainless steel embedding pans were coated with mold release spray and dried in oven at 60°C. On the day of processing, the tissue sample was trimmed to no greater that 1.2 cm in length, and transferred to PBS at 4°C prior to loading it into a labelled cassette. The cord sample was dehydrated in sequential ethanol solutions, each for 30 minutes (50%, 70%, 90%, and 100% x two washes). This was followed by the introduction of xylol in a 50% absolute ethanol/50% xylol solution after which the sample was then placed in absolute xylol for 2 washes of 60 minutes on a rotatory platform with gentle agitation. The spinal cord sections were incubated sequentially in 1:2; 1:1; 2:1 paraffin/xylol solutions in the oven at 55-60°C, taking care not to exceed 60°C, for 1 hour per ratio; this step was also done with gentle agitation. The cord was then transferred to full strength paraffin for one hour, and then to a second batch of full strength paraffin overnight.

The following day, over a heated surface, the sample was removed from the cassette and orientated as desired in embedding pan using a Tissue Tek Embedder (Sakura-Finetek U.S.A, Torrance CA). Paraffin was added to the pan which was placed on a cold surface and left to set ensuring the orientation of the sample was maintained. The appropriate labelled cassette lid was applied over the pan which was then stored at 4°C overnight. Finally, the samples were broken out
of the embedding pans, trimmed of excess paraffin using a heated knife, and the blocks were stored at 4°C until ready to be sectioned.

2.3 Results

2.3.1 Rat Mesenchymal Stem Cells

Cell Morphology

Rat mesenchymal stem cell (MSC) cultures were derived from the bone marrow of femur and tibia from wild type and eGFP transgenic animals (Figure 2.7). The cells available for this project were isolated previously by Dr. Gemma Rooney and by Jarred Nesbitt of the Mayo Clinic. eGFP-MSC were frozen in 2 batches at the Mayo Clinic, with P1 dated 16/11/2005 and P2 30/3/2006, in aliquots of 4 x 10^6 cells per vial. The original yield of the prep from 18 animals was 1.6x10^9 cells plated on T175 flasks at a density of 9x10^5/cm^2. These cells demonstrated a flat, broad phenotype that is triangulated in vitro, characteristic to the cell type, and were used as established cultures up to passage 4.

Figure 2.7: eGFP-MSC as a confluent culture.
MSC Surface Marker Characterization by Flow Cytometry

Prior to the implantation of MSCs into a scaffold model for spinal cord repair, it was necessary to define the cells as mesenchymal stem cells. The isolated MSCs were characterized to meet proposed minimal criteria for defining multipotency (Dominici, Le Blanc et al. 2006). According to the proposal, cells must meet three main qualities based on cell culture, surface marker expression, and their potential to differentiate to mesenchymal cell lineages. For our study,

- Cells were selectively adherent to plastic when maintained in standard culture media.
- Flow cytometry for surface marker and eGFP co-expression demonstrated homogeneous populations of CD90-eGFP, and CD172-eGFP positive cells and a mixed population of CD71-eGFP and CD73-eGFP (66.1 +/- 0.6 %) positive cells (Figure 2.8 left panel).
- eGFP cells were uniformly negative for CD45 pan-leukocyte and CD11b/c macrophage markers (Figure 2.8 right panel).
- Testing for CD105 (endoglin) positive marker was inconclusive as there was significant secondary antibody only control signal on FACS; a suitable CD34 (haematopoetic progenitor) negative marker against rat antigen was unavailable.

MSC differentiation to mesenchymal cell lineage

eGFP-MSC cultures were differentiated to adipose, osteocyte and chondrocyte lineages by protocols established within our laboratory (Rooney, Moran et al. 2008). When cycled through adipogenic induction (3 days) and maintenance media (1 day) for 3 cycles, eGFP-rMCS (Figure 2.9 A) differentiated into adipocytes, as evident by Oil O Red lipid vesicle staining (Figure 2.9 B) and enhanced absorbance of extracted Oil Red O seen in treated cells (Figure 2.9 C). Extracted lystate was from treated cells measured 0.230 +/- 0.022 eGFP-MSC, over untreated controls, 0.089 +/- 0.0 and 0.096 +/- 0.004 (n=3). eGFP-MSCs treated with osteogenic media developed dense mineral deposits seen within the culture monolayer (Figure 2.10 A and B) and contained 3 fold more calcium than
untreated cells (19.85 +/- 0.43 µg/ml control and 55.77 +/- 13.37 µg/ml treated) (Figure 2.10 C).

**Figure 2.8:** FACS analysis using markers as noted to characterize a homogeneous CD90+ population of eGFP-MSC, and a mixed population of CD73+ and CD71 cells. No marker expression is seen for Leukocyte (CD45) or Macrophage (CD11b/c) cell types (key: antigen y-axis shift, gfp x-axis shift). Cells also homogenously positive for cd71, cd172 (Key: 2 batches of MSCs, Red = cells alone; Blue = antigen of interest).

Data regarding chrondrogenesis was presented by Dr. Gemma Rooney (Rooney 2007; Rooney, Moran et al. 2008). Glycosaminoglycan (GAG) concentration ranged from 40 to 65 µg GAG per µg DNA within the cell pellets in treated samples following differentiation.
**Figure 2.9:** Differentiation of eGFP-MSC to adipose lineage. Control MSCs (A) compared to cells following treatment with adipogenic media (1 uM dexamethasone, 200 uM indomethacin, 10ug/ml insulin, and 0.5 mM isobutylmethylxanthine, (B). Cells demonstrate Oil Red O staining, extracted and quantified (C) after 12 days in culture.
Figure 2.10: Differentiation of eGFP-MSC to osteocyte lineage. Control MSCs (A) compared to cells following treatment with osteogenic media (100 nM dexamethasone, 10 mM β-glycerophosphate, 50 mM ascorbic acid 2-phosphate, (B). Cells demonstrate morphologic change and dense calcification. (C) Measurement of calcium levels from extracted control and treated eGFP-MSCs after 20 days in culture.

2.3.2 Rat Schwann Cells

Morphology and S-100 staining

Primary rat Schwann cells were isolated from the sciatic nerves of day 3-5 neonatal pups. The cells were dispersed from nerve fascicles with collagenase and trypsin and plated on laminin coated dishes for 72 hours prior to use. Rat Schwann cells demonstrated a characteristic bipolar, spindle phenotype (Figure 2.11 A-C) and a tendency to align longitudinally for growth in swaths of cells (Figure 2.11 A and B).
The homogeneity of the cultures was assessed with immunostaining for Schwann cell S-100 antigen and with CD90 to identify contaminating fibroblasts. Cultures were measured to consist of 86.2 +/- 4.9% Schwann cells by cell counts. These cells were implanted into the animals within 2 hours of their isolation.

2.3.3 OPF+ Hydrogel Scaffold Fabrication

Positively charged oligo (poly(ethylene glycol) fumarate) (OPF+) scaffolds were fabricated by injection molding and prepared for cell loading. OPF is a PEG based hydrogel incorporating a fumarate moiety. It is made positively charged with the addition of MAETAC to the polymer mix (20% w/w). NVP crosslinks the OPF and MAETAC when reacted with photoinitiator (Irgacure 2959) and UV light at 365 nm (blacklight wavelength). MAETAC is a positively charged quaternary amine. We have shown that the neurite outgrowth from DRGs on the surface of charged OPF disks is
more robust than on neutral OPF, (Dadsetan, Knight et al. 2009) and this was the basis for selecting this material for our in vivo work.

As demonstrated in Figure 2.12, the mold rig is 24.5 mm in length and is a glass tube with an inner diameter of 1.55 mm (A). Teflon plugs for the ends are shown (B), which secure the wires in the correct orientation for parallel channel formation the length of the scaffold column. In C the liquid polymer can be seen within the mold around the wires. The mold is full and without air bubbles which would distort the channel architecture. The mold is then placed under UV light for 30 minutes to polymerize the liquid polymer, and dried overnight at room temperature. The glass tubing can then be slid off the polymer and wires after it shrinks with drying, and this leaves a scaffold tube of 20 mm length that is rehydrated in sterile PBS. The wires slide out easily after the tube swells. The scaffold tube is cut then into 3 mm lengths using a plastic jig.

**Figure 2.12:** OPF+ Scaffold Fabrication. Scaffolds are cast over 7 parallel wires of 290 um diameter, which will yield a final channel size of ~450 um when the polymer is hydrated.
When fully hydrated, the scaffolds measured 3mm in diameter and 2 mm in length, with 7 parallel channels of ~450 μm diameter, or 0.68 μl each in volume (Figure 2.12 D).

**Scaffold fluid volume**

To estimate the fluid volume within a scaffold, fully hydrated 2 mm scaffolds were weighed on a fine balance. After 24 hours of vacuum drying the weight measurements were repeated. Neutral OPF and 20% positively charged scaffolds were analyzed. Neutral scaffolds weighed 5.32 +/- 0.41 mg in their hydrated state and 0.73 +/- 0.11mg when they were dehydrated. Positively charged scaffolds can take up more fluid than neutral ones, weighing 8.31 +/- 0.39 mg in their hydrated state. After the drying process, the weight for neutral and positive charge scaffolds was 0.65 +/- 0.19 mg and 0.58 +/- 0.11 mg respectively. The molecular interactions between the amine moiety in charged OPF with the dipole charge of water may account for the increase in scaffold fluid volume. Converting weight of water to volume uptake, neutral scaffolds contained 5.32 ± 0.41 μl and positively charged scaffolds contained of distilled water respectively 8.31±0.39 μl.

![Scaffold volume](image)

**Figure 2.13:** Scaffold volume depends upon its charge and state of hydration. (A) From left to right is a neutral hydrated, a dehydrated, and a charged hydrated scaffold, with a higher volume noted in the charged scaffolds. (B) Charged scaffolds contain more water by weight given dipole interactions with the positive amine group, n=10 (*** p<0.001).
2.3.4 Schwann Cell and eGFP-MSC Cell Loading onto OPF+ Scaffolds

Sterilizing the scaffolds for spinal cord implantation was a principle concern. Scaffolds were rehydrated in sterile PBS, and then when cut to size were taken through a series of ethyl alcohol dilutions, 100%, 80% 50% and 25% for 20 minutes each prior to equilibrating in autoclaved water and finally in warm tissue culture media for an hour. Scaffolds prepared in this fashion did not grow bacterial or fungal contamination when left in tissue culture media for up to 1 week.

SCs and eGFP-MSCs were suspended in Matrigel at a density of 50,000 cells per µl and loaded into the scaffold channels by pipetting the cell suspension directly onto the open face and drawing through from the opposite face with a dry tissue wipe. The suspension was allowed to set within the channels, and the scaffold was ‘capped’ at each end with either Matrigel or extra cell suspension. As eGFP-MSCs are significantly larger cells than SCs, this determined that density of 50,000 cells/µl be used for direct comparison, instead of 100,000 cells per µl as we have previously reported for SC loaded scaffolds (Moore, Friedman et al. 2006; Olson, Rooney et al. 2009). Control scaffolds contained Matrigel alone.

It was clear in loading the scaffold that their volume would change very quickly when left in the open air to dry, particularly when placed at 37°C to set the Matrigel. Volume would recover equally quickly when the loaded scaffolds were immersed in cell culture media overnight prior to in vivo implantation. We did not note any gaps, particularly around the channel peripheries, secondary to the flux in hydrogel volume around a set volume of Matrigel. We did notice, however, on occasion that air could form beneath the scaffold cap and the channel, as the Matrigel would retract slightly from the level scaffold surface to form a convexity when it dried. In this case, the air was removed by inserting a needle and filling the gap with either additional Matrigel or allowing the gap to fill with cell culture media.
Figure 2.14: Images of scaffolds used for implantation, loaded with SCs (A) and eGFP-MSCs (B). The scaffold has taken up the phenol red from the tissue culture media. Channel size is 450 µm, (blue bar –500 µm) and the channels are seen to be completely filled with Matrigel cell suspension, with a homogenous dispersion concentrated within after the gel has set. A scaffold carries 238,000 cells total into the spinal cord when implanted. After 2-3 days, if left in culture, the MSC cells tended to aggregate within the channel, preferring cell to cell contact within the 3 dimensional space (C) (bar 100 µm).
2.3.5 Surgical Implantation of Scaffolds

Cell-loaded and control OPF+ scaffolds were placed between the rostral and caudal ends of complete spinal cord transections at the level of T9 (Figure 2.15).

Figure 2.15: The surgical procedure in female Sprague-Dawley rats involved a T8-T10 laminectomy (A), to expose the underlying cord, seen here with its dura and dorsal vasculature intact. In (B), placement of the scaffold was between the rostral and caudal stumps of the transected cord. Complete T9 transection was done with a #11 scalpel blade, and ensured using a hooked probe to cut through any residual (usually ventral) attachments. No cord was excised, as the cord springs apart to accommodate the scaffold, and the scaffold did not need to be secured with sutures. The paraspinal muscles were closed tightly over the top and the scaffold sat snugly within the gap. The animal was then reclosed in layers.
2.3.6 Postoperative Outcomes

**Mortality**

Surviving animals were maintained for 4 weeks in the Guggenheim Building conventional animal housing facility at the Mayo Clinic. During the 4 weeks, animals were seen twice daily for bladder expression, for fluid, antibiotic and analgesia administration for the first 5 days post operatively, and assessed for autophagia behaviour, urinary tract infections and motor function recovery.

For the first phase of the project, thirty female Sprague Dawley rats were operated on between December 16\(^{th}\) 2008 and February 24\(^{th}\) 2009. Ten control animals received scaffolds with Matrigel only, of which nine survived for the 4 week time point. An early error was made during the first night after surgery in that rats were housed together in pairs. This resulted in the death of one rat, whose lung was punctured by its pair. From that point onwards, animals were paired following one week of recovery time. Eight animals received scaffolds with Schwann cells, of which six survived. Two animals died from overwhelming sepsis, the first on post-operative Day 5 and the second on Day 9. Ten animals received scaffolds containing eGFP-MSCs, of which seven animals survived the four weeks. Two animals died during the surgery itself, and a third had to be euthanized for severe autophagia of its hind leg. One animal (NM021M) received a scaffold that had been retrieved from NM020M which had just died. Mortality then was 10% for the control group, 25% for the SC group, 33% for the MSC group, and an overall mortality of 20%. Causes of death therefore included excessive intraoperative bleeding, intolerance of anaesthesia, severe autophagia, or post-operative sepsis. When peri-operative and pre-perfusion weights were compared (n=5 of the MSC animals) 2 animals gained weight (17 grams on average) while 3 animals lost weight (14.4 grams on average).

**Autophagia scoring**

Animals which received eGFP-MSC scaffolds displayed a higher degree of autophagia. A novel scoring system was developed to assess this behaviour. 5 animals of each group of the three groups displayed no autophagia behaviour, 2 animals from the Control and MSC groups displayed grade 1 and grade 2 behaviour. The SC group had one grade 3 animal which required sutures to its
abdomen and subsequently died, and the MSC had one grade 4 animal which penetrating injury to the hind limb bone.

**Figure 2.16:** The autophagia scoring system as represented by a histogram of the numbers of animals displaying a degree of behaviour (A) and the average score for each group (B). The scoring system was: 0 points for none observed, 1 point for mild hair pulling or skin exposure, 2 points for biting with fresh blood or skin puncture, 3 points for open wounds and tissue loss requiring sutures, and 4 points for severe wounds requiring that the animal be euthanized. No significant differences in the mean scores were seen with ANOVA means testing.

**Antibiotic use**

Animals were assessed for hematuria and pyuria twice daily, in conjunction with bladder expression and urine volume estimation (0 to 4+). Animals received antibiotics for 5 days post operatively to stem surgery related infections. After this time period, there was concern that animals were developing urinary tract infection secondary to urinary stasis. Initially a protocol was in place whereby an animal would be treated for 3 days twice daily with antibiotics and subcutaneous fluid if the urine was noted to be excessively cloudy or bloody. After the SC group was operated on, a change in protocol occurred whereby the animal would be treated until the urine appeared clear for consecutive 3 days. The
total number of days during which animals in each group was maintained on antibiotics was recorded. Over the course of the experiment, particularly after operating on the MSC group, there was a definite increase in the use of antibiotics (Figure 2.17), such that some MSC animals were maintained for nearly the entire 4 weeks.

![Days of Abx and Fluids](image)

**Figure 2.17:** The number of days animals were maintained on antibiotic and fluid therapy given concern for urinary infection.

Dates of animal surgeries, aftercare observations, autophagia scoring and days on antibiotics for animals NM001 through NM030 are summarized in Table 2.4 in the appendix to Chapter 2.
Basso, Beattie and Bresnahan (BBB) functional analysis

Animals were scored for improvements in hind limb motor function according to the BBB scoring system, at 2 and 4 weeks post-operatively.

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<th>Score (R)</th>
<th>Best leg average</th>
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<th>Score (L)</th>
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**Table 2.5:** BBB Scores for Control (NM001C-NM010C), Schwann cell (NM011S-NM018S) and eGFP-MSC (NM019M-NM030M) animals.
No significant functional recovery was seen, nor were there any differences between groups in their BBB scores. Scores at 2 weeks were 3.81 +/- 0.82 control, 4.00 +/- 0.96 SC and 4.19 +/- 0.70 GFP-MSC animals (Mean +/- SEM). Scores at 4 weeks were 3.81 +/- 0.76 control, 5.0 +/- 0.97 SC, and 3.81 +/- 0.77 GFP-MSC animals.

![Figure 2.18](image)

**Figure 2.18:** BBB analysis shows low scoring consistent with post operative paraplegia and no significant functional recovery over a 4 week interval.

### 2.3.7 Gross Pathology of Spinal Cord Implants

Healthy animals were sacrificed 4 weeks after scaffold implantation. The animals were euthanized by means of deep anaesthesia, and then fixed with transpericardial perfusion of 4% paraformaldehyde in PBS. The vertebral columns removed *en bloc* for gross pathology and histology. After a week of post-fixation, the spinal cords were carefully removed from the vertebral columns (Figure 2.19). The cords were assessed for completeness of transection, for the correct level of implant placement, and scaffold alignment.
At this point the cord was dissected under a microscope, carefully removing the bone and tissue to each side, and using a scalpel blade to cut through lateral nerve roots and to free the underside of the cord from the wall of the vertebral bodies. Rongeurs and mincing scissors were used to break down the overlaying tissue taking great care not to damage the scaffold.

In the Matrigel only control group, 2 animals (NM002C and NM005C) were found to have scaffold implantation into the lumbar spine, and not in the thoracic, and were excluded from the study. The tissue was subsequently used for optimization of antibody staining. One cord had an extensive glial scar (NM007C) but no scaffold was found in the cord (Figure 2.20). We presumed the scaffold had been pushed out dorsally into the musculature and was either broken down, or destroyed during cord dissection. NM003C, NM004C, NM006C and NM008C were found to be fully transected and good specimens for further study. NM004C was an optimal cord and was photographed (please see the series of images in Table 2.6). NM009C was transected approximately 95% the distance of the cord section, and the scaffold was slightly skewed. This sample was processed but put in reserve for analysis if needed. NM010C was fixed in 4% paraformaldehyde + sucrose as a cryoprotected sample for electron microscopy.
Figure 2.20: Control animals NM002C and NM005C had scaffold placement into the lumbar spine due to surgical error during laminectomy. NM007C did not have a scaffold in place at all, but had extensive glial scarring following the transection (arrow).

In the Schwann cell group, only 3 of the surviving 6 animals were found to have scaffolds that were correctly positioned and could be further analysed (NM011S, NM012S and NM018S). During this batch of surgeries, done on December 18th 2008, there were technical difficulties in ensuring that the spinal cords had been fully transected. Consequently, 3 of the animals had incomplete transections, of 60%, 80% and 95% the distance of the cord depth. In each case, there was displacement of the implanted scaffolds. NMO11S is shown here (Figure with 2.21). Scaffolds (NM014S and NM016S) turned perpendicular to the cord within the fibrous capsule surrounding the implant. Interestingly, this was only noted when the scaffold was sectioned, and the channels were noted to be cut in a longitudinal orientation instead of transverse. The scaffold in NM015 was displaced from the cord surface at a 45° angle, and deeply embedded into the surrounding vertebral bone.
With this batch of surgeries, it was learned that an incomplete transection will result in a mechanical apex point at the ventral cord surface, from which the scaffold would be extrude dorsally with cord tension. Scaffolds would be pushed through the laminectomy site, or flip the scaffold 90 degrees within the fibrous capsule. Greater care was taken in subsequent surgeries to be certain that the cord was transected fully and free from any residual attachments between the ends.

In the eGFP-MSC group, 6 animals were found to have scaffolds that were correctly positioned and properly aligned, and were carried forward for immunohistochemical analysis. With this group of animals, a modified method of sectioning was used with 10 slides being cut around the quarter length points. One animal, NM030M was cryopreserved but not processed. An animal that had a slightly skewed scaffold was kept in reserve, and used for antibody optimization.

**Figure 2.22:** A full length rat spinal cord dissected free from the length of the vertebral column, with the scaffold implanted at T9. The cord is backlit to demonstrate the tissue cables within the translucent hydrogel. Rostrum is to the left. Changes to the tissue quality at each end of the cord adjacent to the scaffold are evident as scarring.
Tissue processing results for Control animals

Table 2.6. Summary of the tissue processing outcomes for control animals. 5 spinal cord could be used for further analysis, and 4 animals were excluded from the study. Animals which are highlighted in blue were included for immunohistochemistry.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Group</th>
<th>Polymer Type &amp; Channel Size</th>
<th>Cell Number</th>
<th>Perfusion Date</th>
<th>Processing</th>
<th>Histology Observations</th>
<th>Sectioning Orientation</th>
<th># of Slides</th>
<th>Neurofilament Slide #s</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM002C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>20/02/2009</td>
<td>4% PF</td>
<td>Full cut, Lumbar scaffold placement</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>NM003C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>10/02/2009</td>
<td>4% PF</td>
<td>Full cut, great sections throughout</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM004C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>11/02/2009</td>
<td>4% PF</td>
<td>Full cut, optimal sections, photographed gross</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM005C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>13/03/2009</td>
<td>4% PF</td>
<td>Lumbar scaffold placement</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM006C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>13/03/2009</td>
<td>4% PF</td>
<td>Full cut, good specimen</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM007C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>15/03/2009</td>
<td>4% PF</td>
<td>No scaffold found in the cord</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM008C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>18/03/2009</td>
<td>4% PF</td>
<td>Full cut, good specimen</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM009C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>17/03/2009</td>
<td>4% PF</td>
<td>Slightly skewed, 95% cut</td>
<td>Transverse</td>
</tr>
<tr>
<td>NM010C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>15/01/2009</td>
<td>Frozen storage</td>
<td>4% PF + Sucrose</td>
<td>Cryoprotected sample</td>
<td>Longitudinal</td>
</tr>
</tbody>
</table>
**Figure 2.23.** is a series of photographs of the control animal NM004C, at 3 magnifications (0.8x, 1.2x and 2x) under the dissecting scope. There is good alignment of the scaffold with channels clearly visible, extending between the rostral and caudal stumps, which themselves are scarred and atrophied.

**Tissue processing for Schwann cell animals**

**Table 2.7.** Summary of the tissue processing outcomes for Schwann cell animals. The highlighted animals were included in further

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Group</th>
<th>Polymer Type &amp; Channel Size</th>
<th>Cell Number</th>
<th>Cell Number</th>
<th>Perfusion Date</th>
<th>Sectioning Date</th>
<th>Processing</th>
<th>Histology Observations</th>
<th>Sectioning Orientation</th>
<th># of Slides</th>
<th>Neurofilament Slide #’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM011S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>12/02/2009</td>
<td>4% PF</td>
<td>full cut, good specimen</td>
<td>Transverse</td>
<td>170</td>
<td>64, 83, 99</td>
</tr>
<tr>
<td>NM012S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>15/02/2009</td>
<td>4% PF</td>
<td>full cut, good specimen</td>
<td>Transverse</td>
<td>174</td>
<td>67, 88, 108</td>
</tr>
<tr>
<td>NM013S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>15/02/2009</td>
<td>4% PF</td>
<td>full cut, good specimen</td>
<td>Transverse</td>
<td>170</td>
<td>64, 83, 99</td>
</tr>
<tr>
<td>NM014S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>16/02/2009</td>
<td>4% PF</td>
<td>80% cut, scaffold turned 90 degrees to cord axis</td>
<td>Longitudinal</td>
<td>92</td>
<td>37, 55, 74</td>
</tr>
<tr>
<td>NM015S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>16/02/2009</td>
<td>4% PF</td>
<td>Severe displacement – bone embedded – 60% cut</td>
<td>45 degree</td>
<td>146</td>
<td>46, 65, 85</td>
</tr>
<tr>
<td>NM016S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>24/02/2009</td>
<td>4% PF</td>
<td>95% cut, scaffold turned 90 degrees</td>
<td>Longitudinal</td>
<td>76</td>
<td>20, 37, 57</td>
</tr>
<tr>
<td>NM017S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>15/01/2009</td>
<td>15/02/2009</td>
<td>4% PF</td>
<td>full cut, good specimen</td>
<td>Transverse</td>
<td>178</td>
<td>65, 84, 101</td>
</tr>
</tbody>
</table>
Figure 2.24. (Left) In NM015S, the cord was not fully transected, as can be seen at the underlying ventral aspect. The scaffold was displaced dorsally away from this apex by the elasticity of the cord, through the laminectomy site and into the adjacent vertebral arches and musculature. (Right) The scaffold in NM012S again is well aligned with tissue conduits extending through the channels of the polymer. The scaffold is adjacent to a dorsal root ganglion.
### Table 2.8. Summary of the tissue processing outcomes for eGFP-MSC animals. The highlighted animals were included in further study.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Group</th>
<th>Polymer Type &amp; Channel Size</th>
<th>Cell Number</th>
<th>Cell Number</th>
<th>Perfusion Date</th>
<th>Sectioning Date</th>
<th>Processing</th>
<th>Histology Observations</th>
<th>Sectioning Orientation</th>
<th># of Slides</th>
<th>Neurofilament Slide #'s</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM019M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/01/2009</td>
<td>18/03/2009</td>
<td>4% PF</td>
<td>full cut, good specimen</td>
<td>Transverse</td>
<td>166</td>
<td>72, 88, 104</td>
</tr>
<tr>
<td>NM020M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/01/2009</td>
<td>19/03/2009</td>
<td>4% PF</td>
<td>full cut, perhaps skewed caudally</td>
<td>Transverse</td>
<td>166</td>
<td>83, 97, 109</td>
</tr>
<tr>
<td>NM021M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/01/2009</td>
<td>11/08/2009</td>
<td>4% PF</td>
<td>modified cutting, no % counts</td>
<td>Transverse</td>
<td>36</td>
<td>1, 8, 8</td>
</tr>
<tr>
<td>NM022M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>13/08/2009</td>
<td>4% PF</td>
<td>modified cutting</td>
<td>Transverse</td>
<td>42</td>
<td>4, 4, 4</td>
</tr>
<tr>
<td>NM023M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>14/08/2009</td>
<td>4% PF</td>
<td>modified cutting</td>
<td>Transverse</td>
<td>42</td>
<td>5, 4, 4</td>
</tr>
<tr>
<td>NM024M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>17/08/2009</td>
<td>4% PF</td>
<td>modified cutting</td>
<td>Transverse</td>
<td>42</td>
<td>3, 4, 4</td>
</tr>
<tr>
<td>NM025M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>17/08/2009</td>
<td>4% PF</td>
<td>modified cutting</td>
<td>Transverse</td>
<td>42</td>
<td>4, 5, 4</td>
</tr>
<tr>
<td>NM026M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>18/08/2009</td>
<td>4% PF</td>
<td>modified cutting</td>
<td>Transverse</td>
<td>42</td>
<td>4, 4, 4</td>
</tr>
<tr>
<td>NM030M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>238,000</td>
<td>26/03/2009</td>
<td>frozen storage</td>
<td>4% PFA + sucrose</td>
<td>Longitudinal</td>
<td>42</td>
<td>4, 4, 4</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.25 Fibrous tissue encloses the scaffold in NM021M (left) at the T9 level within the rat spinal column at 4 weeks following placement (dorsal spinus processes removed, rostrum to the left). With the spinal cord dissected free, the scaffold is seen to be well aligned between fully transected ends, the channels are evident, and scarring is seen at the transition areas.
Figure 2.26 (Upper) When fully dissected from the vertebral column, and free from attached tissue and bone, the scaffold is found to be enclosed in a thin fibrous capsule of tissue which is not an extension of the dura. This tissue will be evident on forthcoming histology sections. (Lower) When the capsules is carefully removed, there is a striking translucency of the soft hydrogel, and the orientation of tissue cables extending between the ends of the cord is seen.
2.3.8 The Need for Additional Surgeries

4 control animals of the original 10 surgeries, 3 of 8 Schwann cell animals, and 6 of 10 eGFP-MSC animals were suitable for histologic analysis. We had hoped for eight animals per group at the outset of the project. A second round of surgeries was therefore done on 9 Schwann cell animals on July 2nd 2009 (NM031S-NM040S) and on 4 control animal on July 8th 2009. Of the 9 SC animals, one animal died immediately post-operatively, and a second died from autophagia behaviour on July 4th, leaving 7 animals which survived to perfusion. Of the four control animals, one died of sepsis on July 17th (Day 8 post-op), leaving three animals surviving the four weeks. Initial histologic analysis showed that the surviving Schwann cell animals demonstrated virtually no axonal regeneration. With such poor results, which were inconsistent with all previous results for Schwann cell implantation within the laboratory’s experience, this Schwann cell group was discarded in its entirety. We have not fully analyzed to date the reasons for the failure of the experiment.

To make up the numbers for the Schwann cell group for our project, 3 animals were used at random from a parallel study within the lab. This study used Schwann cell loaded scaffolds, at densities of 100,000 cells per ul Matrigel, within 4 different polymer types implanted into the thoracic transection model. Axonal regeneration was compared using neutral OPF, positively charged OPF, poly(caprolactone fumarate) (PCLF) and poly(lactic co-glycolic acid) (PLGA) as the polymer substrate. Most of the original studies from our lab had used PLGA scaffolds, and this was therefore used as the benchmark for the polymer comparison study (Chen, Knight et al. 2011). I had assisted with the scaffold loading, surgery, and animal aftercare for these animals. Three animals, P7, P44, and P45 from within the positively charged OPF group were randomly selected for inclusion in our cell-comparison study.
The animals included therefore in the histologic analysis to follow, for an n=6, are summarized in Table 2.9:

<table>
<thead>
<tr>
<th>Control</th>
<th>Schwann cell</th>
<th>eGFP-MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM003C</td>
<td>NM011S</td>
<td>NM019M</td>
</tr>
<tr>
<td>NM004C</td>
<td>NM012S</td>
<td>NM023M</td>
</tr>
<tr>
<td>NM006C</td>
<td>NM018S</td>
<td>NM025M</td>
</tr>
<tr>
<td>NM008C</td>
<td><strong>P7</strong></td>
<td>NM027M</td>
</tr>
<tr>
<td>NM041C</td>
<td><strong>P44</strong></td>
<td>NM028M</td>
</tr>
<tr>
<td>NM044C</td>
<td><strong>P45</strong></td>
<td>NM029M</td>
</tr>
</tbody>
</table>

spare: NM009C spare: none spare: NM021
2.4 Discussion

2.4.1 MSC Characterization using Surface Markers

This chapter has described methods and results for rat MSC isolation and characterization. It may have been possible to use MSCs from other species, including human and mice. It has been reported that human MSCs are well tolerated and actively therapeutic in rodent models of stroke (Chopp and Li 2006). MSCs can also be isolated from many tissues other than the bone marrow, including peripheral blood, and umbilical cord blood and Wharton’s jelly, adipose tissue, amniotic fluid, along with other bone sources including periosteum, synovial fluid and membrane, and articular cartilage. There is growing evidence that there are significant differences in the phenotype and overall biology of the cell, dependent not only upon the tissue of origin but perhaps even the clonal population of a given isolate (De Bari and Dell'acco 2008).

It was an essential part of the thesis to demonstrate clearly that the implanted cells were indeed the cell types that we intended to implant. We chose to isolate MSCs from the same Sprague-Dawley strain as the surgeries would be performed in, with the difference being of course that MSCs were isolated from eGFP transgenic rats. The MSC isolation technique involved plating of whole marrow flushes. In isolating MSCs from the bone marrow, contaminating cells would consist of marrow stroma, cells involved in bone function (osteocytes, osteoclasts, osteoblasts) and blood cells of all myelocytic and lymphocytic lineages including haemopoetic stem cells.

The method of isolation relied upon differential adherence to the tissue culture plate and the rapidity with which MSCs out-propagate other cells therein to develop the culture. No unique markers can unequivocally identify an MSC and differentiate it from another cell type (Augello, Kurth et al. 2010). Some labs are developing micro-array or PCR based methods for genetic screening and characterization of MSC cultures (Kawauchi, Terasaki et al. 2010). In practice most are using the presence or absence of CD cell surface markers for identification, some even for cell-sorting isolation for large scale clinical use.
Axonal Regeneration Supported by OPF+ Cell-loaded Hydrogel Scaffolds (Spiropoulos, Theodosaki et al. 2011). We relied on the position statement of the International Society for Cellular Therapy (Dominici, Le Blanc et al. 2006) in deciding which criteria and which surface markers would ‘qualify’ a cell to be considered as an MSC. The statement was developed to be an international consensus of what minimal criteria should be used to define human multipotent mesenchymal stromal cells, given inconsistent methods, source tissue, defining characteristics among laboratories. Essentially, three criteria were proposed:

- Cell adherence to plastic in standard tissue culture conditions (which is prerequisite to establish the culture in vitro and has long been a defining trait (Caplan 1991))
- Specific surface antigen phenotyping, which included
  - cultures being ≥ 95% positive in cells expressing CD105, CD73 and CD90,
  - cultures being ≤ 2% positive in cells expressing CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR.
- In vitro differentiation to osteoblast, adipocyte and chondroblast cell types.

The cells require positive surface marker expression consistent with the phenotype and the absence of markers found on contaminating haematopoietic cells. The subsets of negative expression include the pan-leukocyte marker CD45, the haematopoietic progenitor marker CD34, monocyte and macrophage markers CD14 and CD11b, and B-cell specific markers CD79a and CD19. Only one of the macrophage and B-cell antigens needs to be tested. The HLA marker should be absent unless the cells were stimulated by gamma interferon.

The proposal was useful in that it established a clear framework of three guiding principles for cell identification. It was not useful in that the criteria only applied to human MSCs. Phenotyping the surface marker expression to identify MSCs has been extensively developed in human (Jiang, Jahagirdar et al. 2002) and mouse models (Tropel, Noel et al. 2004). Phenotyping in rats has been relatively delayed, and there is no consensus as to which markers are required for characterization. We found that many of the suggested markers for human MSC criteria were not available with reliable monoclonal or quality-control tested antibodies. It has been noted that even among mouse strains, there are differences
in marker expression and behaviour in culture (Fiorina, Jurewicz et al. 2009). It has further been argued that the criteria are overly dependent upon cell culture conditions, further removing the cells from their native phenotype.

Renewed efforts are being made to develop a marker set for large scale clinical (GMC-grade) MSC isolation that would include both negative and positive selection attributes. Interestingly among the candidates is a neural marker, the low affinity growth factor receptor (LNGFR) (CD271) and the platelet-derived growth factor receptor (PDGFR, CD 140b) found to be consistently expressed by MSCs. Others may include novel epitopes including the namesake mesenchymal stem cell antigen-1 (MSCA-1) and CD56, a marker for natural killer, neural, and muscle cells (Battula, Treml et al. 2009).

Relatively poor antibody availability for rat epitopes, along with with cost constraints led to a limited analysis in our work. We therefore focused on what we could see as the most commonly used markers in rats and what was available as reliable, equivalent rat antibodies. Our results have shown homogeneous populations of cells positive for CD90, mixed populations of CD71, CD73, and negative marker expression for CD45, CD11b. This data was generated on cells at passage 2, as a measurement of the overall batch. A recent study done in conjunction with the University of Minnesota detailed the immunophenotype profile of rat bone marrow derived MSCs over 10 passages (Harting, Jimenez et al. 2008). The study found that the MSC culture would not become homogeneous in its surface marker expression until passage 4. Until that time, mixed populations of cells were observed: CD11b and CD45 were found on 35% and 75% respectively of cells assessed at P0, falling to less than 2% each at passage 4 while CD73 and CD105 were found on less than 20% of cells initially, peaking in their expression between P4 and P6. CD90 expression, perhaps the hallmark of the MSC phenotype, could only be seen in 25% of P0 cells and in an expanded, uniform population by P3. Other positive markers, including CD49e, Stro-1, and CD29 (β-integrin) similarly needed 3-4 passages to become homogenous.

Consistent with our own observations, by late passages P8 to P10, surface marker expression was seen again to become highly variable. Another recent analysis directly compared surface marker antigens on P2 and P7 MSCs across 4 rat species, Sprague Dawley (as used in our experiments), Fisher, Lewis and Wistar species (Barzilay, Sadan et al. 2009). The positive markers used were
CD90 and CD29, and negative markers included CD45, CD11b, and MHC class II. The different strains exhibited equivalent cell surface phenotypes, with similar rates of cell expansion and differentiation capacity.

In regards MSC isolation from transgenic rats, (Harting, Jimenez et al. 2009) have shown rapid decline in eGFP expression within the MSC population as the cells are passaged. Whereas 87.5% of confirmed MSCs were GFP positive by flow cytometry at P0, by P1 the proportion had fallen to 44.1% where it remained stable around the 50% mark through passage 8. Equivalent results were seen with microscopic visual cell counts using fluorescence and eGFP antibodies. The proportion remained stable following cell differentiation to adipocyte lineage.

It is therefore probable that what we see in our isolate is a snapshot of a cell population in flux, still establishing an equilibrium of surface marker homogeneity particular perhaps to the rat strain, perhaps even to the clonal origin of the particular isolate. Ultimately it is unclear what exact phenotype range was put into the scaffold, as the P2 cells used needed to be expanded into high numbers for scaffold loading, and would have had passage equivalents in large flasks. Also, various vials of cells would have been used in different time frames of the animal surgeries. Judging from the work cited above, by passage 3 or 4 the cell population should have stabilized at the time of implantation. We could not know what effect the *in vivo* environment, particularly in regards to the early inflammatory phases, would have on the distribution of phenotype or how this may influence phenotypic change. Finally, our cells at P2 did show a homogeneous expression of eGFP. That the percentage of cells expressing eGFP at later cell divisions might have approached 50% would certainly complicate our attempts to track cell fate and assess the influence of cells surviving at 4 weeks *in vivo* (Chapter 3).

### 2.4.2 MSC Characterization using Cell Differentiation

Differentiation of rat eGFP-MSCs was demonstrated in our study to each of the adipocyte, osteocyte and chondrocyte lineages. Osteogenic differentiation utilized incubation of cell monolayers in media containing ascorbic acid, β-glycerophosphate and dexamethasone, and measurement of calcium deposition, as described in the classic Science paper by (Pittenger, Mackay et al. 1999).
Adipogenesis utilized treatment with 1-methyl-3-isobutylxanthin, dexamethasone, insulin and indomethacin with Oil Red-O staining of lipid vacuoles. Chondrogenesis was more complicated, requiring prolonged incubation of pelleted cells with transforming growth factor-β and measurement of increased proteoglycan or collagen type II concentration. In our hands, there is not homogeneous differentiation of the MSC cell culture to the desired lineage upon appropriate treatment, as only subsets of the culture are seen to exhibit the new phenotype in each case. This suggests that only small clonal populations within the cell mass have a variable potency to transform. When colonies were derived from single cell clones in Pittenger’s original work, all 6 colonies underwent osteogenic differentiation, five underwent adipogenic differentiation, and two also underwent chondrogenic differentiation. Some colonies therefore possessed a multipotent capacity for lineage differentiation and some colonies displayed a limited differentiation under these experimental conditions.

No attempt was made to differentiate MSCs into neuronal phenotypes prior to their implantation. When MSCs were differentiated into neurons and injected into a cervical microwire injury model, the cells were seen not to maintain neuronal phenotypes (Lu, Jones et al. 2005). Such ‘transdifferentiation’ was the subject of Dr. Rooney’s PhD thesis from REMEDI (Rooney 2007). Many studies have reported that in response to (often harsh) chemical and physiologic stimuli, as well as genetic induction, MSCs can become cells with glial or neuronal phenotypes, at least in terms of expressing lineage specific markers. This has remained a controversial area, in that the phenotype may indeed be restricted to being what the cells look like as opposed to having any true neurologic capacity. In vitro, phenotypic changes in response to rather harsh chemical stimuli (β-mercaptoethanol or dimethyly sulfoxide) has been described to occur in a matter of hours, rather than a gradual process as would be expected with genetic cues. The new dendritic-like cell processes seen are possibly the result of cell shrinkage and intensification of neuronal marker staining through volume loss. The transduced cells have for the most part lacked any functionality, such as myelin formation or electrophysiologic activity. In vivo, the transdifferentiated cells may have fused with innate cells, providing immunostaining positivity (Lu, Blesch et al. 2004). In practical terms, we have taken the approach that it was not necessary to further investigate the potential for neuronal lineage in this model.
Our scope rather was to investigate phenomena that are better understood, such as their immuno-modular potential or their ability to be genetically transduced as a cellular delivery system.

2.4.3 Schwann Cell Isolation Techniques and their Application

Dr. Windebank’s laboratory has been using isolated Schwann cell preparations since the early 1980s and lab notebooks from this time were an interesting and valuable resource in the further development of this technique. Around this time the cells were isolated for myelination experiments of peripheral nerve preparations. The technique has been involved for spinal cord work and applied in the context of the PLGA and OPF scaffold placement, including the comparison of optimal cell types (Olson, Rooney et al. 2009) and polymer types.

Schwann cells have been consistently shown to be one of the most effective therapeutic cell types in transplantation and regeneration after experimental spinal cord injury (Xu, Zhang et al. 1999; Oudega, Gautier et al. 2001; Pinzon, Calancie et al. 2001; Bunge 2002; Takami, Oudega et al. 2002; Pearse, Pereira et al. 2004; Hurtado, Moon et al. 2006; Novikova, Pettersson et al. 2008). These cells reduce the size of spinal cysts, remyelinate axons (Chen, Knight et al. 2009) and improve functional recovery in spinal cord injury (Biernaskie, Sparling et al. 2007; Lavdas, Chen et al. 2010). They have also been shown to produce a number of growth factors that initiate and support the growth of axons, and that these cells express cell adhesion molecules on their surface for axon guidance (Papastefanaki, Chen et al. 2007).

The cultures are in used as the primary culture within 2-3 days of harvest. Culture composition was assessed with S100 staining, and again as we saw with the MSC preparation, to some degree a heterogeneous culture was implanted. The proportion of fibroblasts contaminating the Schwann cell culture was on the order of 10-15 percent. This proportion seemed to be directly related to the skill with which the fibrous perineurium could be stripped from the sciatic nerve pieces leaving behind the fascicles to be digested enzymatically. To that end I am very grateful to Jarred Nesbitt, who taught the technique given many years of experience. As we will see in chapter 4 some modifications to the culture
technique were used to have a more durable culture, to allow time for retroviral transduction and cell selection for growth factor secreting cell lines.

One of the most disappointing aspects of this project was the failure of a Schwann cell preparation to support any axonal regeneration in a group of 7 animals. We were unable to discern any one change in the culture preparation, the scaffold chemistry or preparation, implantation technique, animal aftercare or survival that could have contributed to failure. One observation perhaps of significance was that the scaffolds were particularly difficult to extract postmortem from the spinal canal. The scaffolds were firmly adherent to the surrounding tissue and on gross inspection there seemed to be more fibrous tissue formed embedding them than in the other animal groups including prior Schwann cell groups. This might imply a higher degree of inflammation or perhaps even graft rejection and thus poor biocompatibility within this particular group, for reasons unknown. It was suggested by one member of our group that the low axon counts might have been related to the length of time the tissue was left in the fixative. However I strongly disagreed with that suggestion given (as we will see) scaffold sections have a very useful internal control for Schwann cell and axonal staining in dorsal root ganglia. In the limited number of sections we reanalyzed, the DRG's seen in cross-section were staining appropriately. To continue the cell characterization within channels (Chapter 3) slides from three animals implanted with OPF+ Schwann cell scaffolds as part of the polymer comparison study were used. We felt this was an appropriate substitution despite the differences in cell numbers loaded.

2.4.4 OPF Hydrogels

OPF is a novel polymer developed by our collaborative group. Within the lab there has been an evolution of the materials used. Rapid progress in hydrogel chemistry has produced materials more suitable for the spinal cord in respect to their mechanical properties. Highly hydrated and soft polymers have similar properties to spinal cord tissue. In this thesis and in our comparative study (Chen, Knight et al. 2011), we demonstrated that OPF and OPF+ contains a large amount of water that decreases the flexural and compression modulus of these materials to values similar to fresh spinal cord tissue. PLGA and PCLF have a higher degree
of rigidity, which may contribute to scaffold displacement, mechanical damage to the cord ends, gaps in their placement and cyst formation at the cord junctions. In our experience the OPF polymer also has a predictable degradation profile in vivo, and we did not observe any increased inflammatory response compared to an empty cage and PLGA control groups (Kim, Dadsetan et al. 2010). With a highly porous three-dimensional structure these materials can provide ingrowth for neural tissue as well as improved cell adherence with a positive charge (Vroemen, Aigner et al. 2003). Positively charged OPF improved primary sensory neuronal attachment and myelination to sustain the primary nerve cells and a glial environment (Dadsetan, Knight et al. 2009).

2.4.5 Advances in Surgical Technique and Animal Care

Along with advances in the materials used, this study has shown the importance of optimizing surgical technique for scaffold placement, perioperative anesthesia, and postoperative animal care. Few studies have shown a high degree of polymer integration with the surrounding tissue (Teng, Lavik et al. 2002; Bunge and Pearse 2003; Prang, Muller et al. 2006; Stokols and Tuszynski 2006), as there have been difficulties with containment of the cell types introduced within the lesion cavities, formation of cysts which themselves become obstacles for axonal regeneration, as well as discontinuity or rupture of the growth conduit. To improve the integration of implants we have modified the surgical technique to minimize the overall trauma of the transection procedure. Such modifications have included smaller laminectomies, smaller dural incisions, a quick and precise cord transection with rapid hemostasis from ventral and dorsal artery bleeds, accurate placement of the scaffolds within the appropriate sized gap and ensuring good alignment prior to firm closure of the overlying paraspinal muscles. Incomplete transection of the cord was particularly problematic, as the intact ventral tissue served as a mechanical fulcrum to displace the scaffold dorsally.

With regard to animal aftercare, novel procedures were developed to monitor the animals for postoperative infection, not only at the wound site but also with regard to systemic infection. A grading system was developed to assess urinary output and hydration status, and a new scale was developed to assess the animals for pain-related behaviors such as odynophagia. Use of antibiotics was
slightly controversial within our lab group as several animals, particularly in the MSC group, were on antibiotic therapy nearly for the duration of their experimental endpoint. Several factors contributed to overuse of antibiotics, including the decision to wait until urine remained clear for 72 hours before discontinuing therapy. Not every instance of clouded urine, particularly if it only involved the initial stream, would have related to an acute infection. In these cases antibiotics were often initiated. We do not have any idea what effects the use of fluoroquinolone antibiotics would have on the regenerative environment.

Having said that, these changes clearly improved the overall welfare of the animals in the postoperative period, and probably contributed to higher numbers of animals completing the time course for the experiments in relative good health. Early on in the work we attempted to pair the animals together immediately after surgery. Unfortunately there was an incident where one animal mutilated and punctured the long lung of the second. Animals were subsequently paired one week after the surgery without further incident and with improvements in the welfare of the animals including their grooming and level of activity.

2.4.6 Lack of Functional Improvement

Functional recovery was not seen in any of the animal groups at both 2 and 4 weeks, as assessed by the BBB locomotor scale. We hypothesized that a certain threshold number of regenerated axon numbers would be required to exert a significant, detectable functional recovery after the injury in this time frame, within the specificity of BBB scoring. Clearly, not all traversing the scaffold axons reach and connect to target neurons correctly and functionally. Indeed we have observed variability in the direction of axonal growth that was dependent upon the polymer type (Chen, Knight et al. 2011). As we will see in the next chapter, OPF with positive charge may play a role in linearizing growth to a confined central core within the channel. A four week time frame is insufficient to observe functional recovery following complete transection. We have also recently demonstrated by axon tracing studies that regenerated axons through Schwann cell loaded PLGA scaffold extended for up to 15 mm beyond the scaffold into the distal cord. However, even after 2 months functional improvement was not observed (Chen, Knight et al. 2009). The negative results in
functional assessment are also consistent with our previous studies utilizing a transection model of scaffold placement (Olson, Rooney et al. 2009).

2.4.7 Conclusions Regarding MSC Implantation

It has been seen then that a heterogenous population of mesenchymal stem cells was loaded into OPF+ scaffolds and implanted into the transected cord. Prior to further cell expansion and implantation, we utilized negative surface marker criteria to minimize the risks of contamination with hemopoetic cell lineages, and demonstrated a mixed population with regards to positive surface marker selection. Cell differentiation to mesenchymal lineages was achieved towards lipogenesis, chondrogenesis and osteogensis \textit{in vitro} at an early passage and in a subclonal population of the culture. The influences defining our cell population included the animal and its strain, the tissue source, the passage at which the cells were analysed, the availability and cost of suitable antibodies to rat antigens, and the comparison to criteria defining human MSCs in the absence of robust criteria for the rat. It is not possible to know given our experimental design, how the phenotype or the differentiation potency may have changed within the environment of the injured cord. Nor were we interested in the transdifferentiation of the MSC into neuronal phenotypes either pre or post implantation.

A cell population as heterogeneous as one selected by adherence and diluted by cell doublings may not be an ideal starting point. Future work with MSC cultures may require more standardized means to select populations with new markers using cell-sorting techniques or perhaps the use of established clonal lines. This would be critically important both in terms of defining a more stable model by which the therapeutic effects of MSCs in spinal cord repair could be teased out, as well as for the standard requirements of cell culturing for clinical application in patients.
Chapter 3:

Quantifying Scaffold Channel Architecture by Immunohistohistochemistry and Stereology

3.1 Introduction

Without question, spinal cord transection is a severe injury to impose upon the model animal. Many have argued it has no clinical correlate in human patients given how rarely the cord is lacerated. It has been our view that many patients with spinal cord injury (up to 40%) have the functional equivalents of a transection injury, given the density of their neurologic deficit and lack of recovery potential. Implantation of OPF scaffolds across the injury field could serve as a bridge to facilitate or guide regenerating axons across such injury sites for recovery of function (Papastefanaki et al., 2007). We have proposed that the short distances needed to bridge the cord to improve respiratory function may have positive impact on patient morbidity and mortality.

For the scientist the issue also speaks to the development of a model, whereby individual facets of the repair process can be meticulously separated free and perhaps controlled within the complexity of the injury response and regeneration. It may be necessary to isolate the regenerating environment from intact functional cord tissue, such as would be present in a hemisection model, or the complex responses occurring in contusion models. With isolation one may begin to see fundamental trends or principles from which an understanding can first arise, followed by the means to intervene based upon that understanding. Our proposal has been that cell-seeded polymer scaffolds in a transection model allow for separate and controlled manipulation of the micro-architecture, surface properties, and the molecular and cellular micro-environment of the regenerating spinal cord. The ability to control these variables with precision may enable the scaffold implant to be informative about individual facets of the repair process.

In 2004 the lab published a comparison of spinal cord injury models (Talac et al., 2004), detailing several advantages of the complete transection
model. These advantages included the certainty and consistency with which the animal's complete functional deficit is achieved. In contrast to a hemisection model or contusion model, complete transection minimizes the influence of nervous tissue adjacent to the injury that could either still function or contribute to the regeneration. For transection the latter would be of concern for the caudal and rostral ends and associated structures such as the DRGs. An advantage of hemisection is in animal aftercare, whereby the animals maintain bladder functioning, and overall the animal regains lost neurologic function more quickly. However the actual injury is variable, depending on the depth of cut, or if and how the dorsal section of the cord is removed etc. A further advantage of the scaffold transection model was how readily one could assess the anatomic continuity of regenerated axons by microscopy.

In this chapter, we begin to show aspects of the scaffold model whereby axon counts can be seen in relationship to other influences in the regenerating environment. Detailed immunohistochemistry and stereology approaches are applied here to the model across the three types of scaffolds implanted containing Matrigel, Schwann cell and MSCs. We look first at the scaffold as a means to make observations of value in the spinal cord. The tissue architecture is examined by means of a general pathology overview, and then an analysis of the cell types that are contributing to structural and functional compartments within the scaffold channels. Quantification of axonal counts is seen now in relationship to the developing channel vasculature.
3.2 Materials and Methods

3.2.1 Tissue Sectioning of Paraffin Embedded Samples

Prior to sectioning, the section thickness, planned sampling regions and the section orientation (transverse or longitudinal for allocated animals) were determined. The sections were cut as 8-µm-thick serial sections using a Reichert Jung tabletop microtome (Leica, Heidelberg, Germany).

Scaffold quarter interval and staining record

A sectioning information sheet was developed for every animal to track the sample and how it was cut. A sheet detailing the sectioning of a representative animal (NM004, Matrigel Control) is shown on the following page. At the top, the sheet identifies several key parameters:

- the animal,
- the time point of animal harvest,
- the scaffold material,
- the channel size,
- the date of sectioning,
- the thickness of the sections,
- the total number of slides generated,
- general comments about the sample including the transection efficiency and scaffold alignment.

The middle section of the form provides details of how the sample was cut. Beginning at the angled rostral end of the spinal cord, slides #1 – 29 contained 10 sections per slide and had tissue from the rostral intact cord without scaffold material. At the proximal scaffold-cord interface, slides #30 – 43 had 5 sections per slide. Once well into the scaffold itself, slides #44 – 140 each had 3 sections per slide, followed by the distal transition into the scaffold-cord interface (again 5 sections per slide, slide #141-164), and then into intact caudal cord without scaffold material, 10 sections per slide, slide #165-176.
Sectioning Information Sheet

<table>
<thead>
<tr>
<th>Date:</th>
<th>2-11-09</th>
<th>Scaffold Material:</th>
<th>OPF+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal ID:</td>
<td>NM004</td>
<td>Orientation:</td>
<td>Transverse</td>
</tr>
<tr>
<td>Time-point:</td>
<td>1 month</td>
<td>Section Thickness:</td>
<td>8 micron</td>
</tr>
<tr>
<td>Channel Size:</td>
<td>~450 micron</td>
<td>Total Slides:</td>
<td>176</td>
</tr>
<tr>
<td>Cell Type:</td>
<td>Matrigel Control</td>
<td>Other Comments:</td>
<td>Complete transection, good alignment. Everything Collected</td>
</tr>
</tbody>
</table>

Spinal Cord

Rostral Intact Cord: 10 Sections/Slide
Rostral Interface: 5 Sections/Slide
Scaffold: 3 Sections/Slide
Caudal Interface: 5 Sections/Slide
Caudal Intact Cord: 10 Sections/Slide

<table>
<thead>
<tr>
<th>Scaffold Position</th>
<th>Represented by Slide Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Quarter</td>
<td>#69</td>
</tr>
<tr>
<td>One Half</td>
<td>#93</td>
</tr>
<tr>
<td>Three Quarters</td>
<td>#117</td>
</tr>
</tbody>
</table>
Having cut the sample and recorded in this manner, it was then possible to calculate which slides represented the 1/4, 1/2, and 3/4 distances through the scaffold and to also identify which immunohistochemical stains were used for each slide number within these areas. For animal NM004, the quarter, half and three quarter slide numbers were #69, #93 and #117 respectively. The calculation made was to divide the scaffold distance (slide #44 through #140, or 96 slides) into quarters (96/4 = 24 slides), and adding 24 in sequence to slide number 45, yielding the first quarter length slide to be #69. The half way point was 96/2 plus 45, = slide #93, or alternately to add 24 slides onto the quarter length number, 69 + 24 = #93, and so forth. The form details that Neurofilament staining was done at slide numbers 70, 92, and 111, adjacent to the quarter length slide numbers. Each slide for subsequent stains was selected from these areas and recorded, here for example listing Von Willebrand factor (slides 71, 91, and 112), CD45 (slides 69, 90, 116) GFAP (slides 72, 90, 115), S-100 (slides 68, 93, 113) and Vimentin (slides 75, 94, and 118). This process of recording was repeated for each animal sample sectioned and analyzed with immunohistochemistry staining.

For 6 of the eGFP-MSC animals, a modified sectioning protocol was used, to focus on the areas of interest within the scaffold, and to reduce the burden of work that was involved in fully sectioning through each scaffold. Instead of sectioning the through the scaffolds and keeping each portion as a slide, usually in excess of 150 slides worth of tissue, 10-12 slides were cut and kept at each of the ¼, ½ and ¾ distances of the scaffold. This was accomplished by sectioning through the scaffold and keeping account of the distance through the scaffold that had been cut, knowing each section was 8 microns in thickness, but discarding those sections that were not in the areas calculated to be at quarter lengths. The calculation of quarter lengths was based upon all scaffolds previously sectioned in full, as the average distance from when the scaffold-cord interface was first seen on a section to first quarter length point. Slides for these animals were therefore labelled different, and being ¼ slides 1-10, ½ slides 1-10, and ¾ slide 1-10, with the Neurofilament staining typically being done on slides numbered as 4 or 5 in that series.
3.2.2 General Procedures

Deparaffinization and tissue rehydration.

Once the slides in each area of interest were selected at quarter scaffold lengths, each was carefully labelled and processed for staining. Slides in batches of 20 were warmed in a 50°C oven for 3 hrs to overnight to soften the paraffin, which was dissolved away from the tissue in two changes of xylol for 10 minutes each. The tissue was then rehydrated in descending concentrations of ethyl alcohol, 100% x 2 washes for 5 minutes each, 95% x 1 wash, 80% x 1 wash, 50% x 1 wash and then into 2 changes of pure distilled water to remove the alcohol.

Haematoxylin and eosin staining

To study the general architecture of tissue generated within scaffold channels, sections were stained with Haematoxylin and Eosin (H&E) or Mason’s Trichrome staining. For eosin autofluorescence studies, a dedicated group of slides was stained with hematoxylin and eosin across each of the animal groups and at each scaffold level. This procedure involved de-paraffinizing the slides in xylol and rehydrating the tissue in graded alcohol dilutions, as described above. Once the slides were rinsed fully hydrated in water, they were passed through a standard sequence of solutions set up in the following order in glass universal containers: Haematoxylin (purple) (Sigma-Aldrich) for 5 minutes, Tap Water rinse (30 seconds), Differentiating Solution (210 ml 96% ethanol, 70 ml ddH2O, 3 ml concentrated HCl) for 5 minutes, Tap Water rinse, Eosin (blue) (Sigma Aldrich) for 3 minutes, followed by 2 rinses with tap water changes.

Mason’s Trichrome staining

With this method, a combination of three dyes was used to stain different tissue components. Collagen is identified in blue, cell nuclei in black, and cytoplasm and keratin are seen in tones of red. Sections stained with Mason’s Trichrome were deparaffinized and rehydrated in graded alcohol washes and maintained in distilled water. The slides were then immersed in the first stain, Weigert’s Iron Haematoxylin working solution (equal parts of Haematoxylin 1 g/100 ml 95% ethanol and 4 ml 29% ferric chloride, 1 ml concentrated HCl to 100 ml in distilled water) for 10 minutes. Slides were rinsed with warm tap water for
10 minutes prior to being immersed in the second stain, Biebrich scarlet-acid fuschin solution for 15 minutes (90 ml 1% aqueous Biebrich scarlet, 10 ml 1% aqueous acid fuschin, 1 ml glacial acetic acid). After rinsing, the slides were differentiated for 15 minutes in phosphomolybdic-phosphotungstic acid solution for 15 minutes (equal parts 25 ml 5% phosphomolybdic acid and 5% phosphotungstic acid), or until the collagen portions of the section were no longer red. The sections were briefly transferred without rinsing to aniline blue solution for 5-10 minutes (2.5 g aniline blue and 2 ml glacial acetic acid to 100 ml in distilled water). Final colour differentiation was with 1% acetic acid solution for 2-5 minutes.

**Tissue dehydration and slide mounting.**

When the process of staining was completed, the tissue was dehydrated again and mounted for preservation and microscope imaging. Dehydration was done with increasing concentrations of ethyl alcohol rinsing the sections gently for 5 minutes in 50%, 80%, 90%, then 2 changes of absolute alcohol prior to 2 changes of xylol. The slides were then coverslipped and mounted using a xylol based mounting media (DPX, Sigma-Aldrich), drying the slides overnight at room temperature in a fume hood.

### 3.2.3 Immunohistochemistry for Paraffin Embedded Tissue

The specific cellular structure within scaffold channels was analyzed by antibody immunohistochemistry. Following deparaffinizing and rehydrating the sample sections, the tissue was incubated for 5 minutes in PBS with 0.4% Triton X-100 (Sigma-Aldrich) on a rotatory shaker, to permeabolize and prepare it for the antigen retrieval step.

**Antigen retrieval.**

The processes of paraformaldehyde fixation, paraffin embedding and ethanol dehydration of tissue can result in structural alternation of protein antigens including cross-linking, such that they will not be as readily bound by antibody. Antigen retrieval has been developed as a step in immunohistochemical staining to improve antigen detection (MacIntyre, 2001). Antigen retrieval was carried out
in a rice steamer apparatus. A fresh batch of citrate buffer solution (10mM solution of sodium citrate, with 0.05% Tween 20, pH 6.0) was made using 2.94 g tri-sodium citrate (dihydrate) in 1000 ml distilled water, adjusting the pH to 6.0 with 1N HCl and adding 0.5 ml Tween 20 (Sigma-Aldrich). The buffer was prewarmed for 20-30 minutes to 96ºC by placing the slide container with buffer in a small water bath within the enclosed rice steamer. When the temperature reached this point, the slides were transferred from the PBS wash directly into the hot citrate buffer and incubated for 20 minutes. The slides were subsequently cooled while still in the citrate buffer in a second water bath with running cold water, and then rinsed in two 5 minute washes of PBS with 0.1% Triton X-100.

For stains that involved Peroxidase-Streptavidin reactions, endogenous peroxidase was blocked at this stage in the process, by incubating the tissue in 1.5% hydrogen peroxide in PBS for 20 minutes. If the stain involved using a fluorescent marker, this step was omitted. The slides were then washed 3 times for 5 minutes in PBS with 0.1% Triton X-100 prior to blocking.

**Blocking**

Blocking against non-specific protein binding was performed for 1 hour using a mixture of commercially available BSA reagent (Power Block™ Universal Blocking reagent 10X (Biogenex, San Ramon, CA)) with 8% normal goat serum in PBS with 0.4% Triton X-100. Slides were removed from the previous PBS wash and the circumference of the tissue section was wiped dry with paper towelling, leaving a rectangle of liquid. This technique enabled smaller quantities of blocking solution and then antibody solutions to be used as the applied liquid would remain within the circumscribed area by surface tension. About 100 ul blocking reagent was needed per slide. Prior to applying the primary antibody, the slides were briefly rinsed in PBS with 0.1% Triton and rewiped.

**Primary antibodies**

Primary antibodies are listed in Table 3.1 in the appendix to Chapter 3. Antibodies were selected on the basis of demonstrated, published use in paraffin-embedded sections as detailed in the appendix. Primary antibodies were diluted as indicated in a commercial antibody diluent with background reducing components (Dako, Carpinteria, CA). The solution is proprietary but contains polyethylene
glycol and Trizma base with 15 mM sodium azide. Diluted primary antibody was applied to the sections allowing for 80-100 ul per slide, following which a small piece of Parafilm™ cut to size was placed over the solution prior to incubating the slides in a humidified slide chamber overnight at 4ºC. The Parafilm proved to be a very effective method in stopping the incubating slides from drying out and limiting antibody run-off if the shelving was uneven or the slide box was moved. The following day, the Parafilm was gently removed with forceps and the slides washed 3 times for 5 minutes in changes of PBS with 0.1% Triton X-100.

**Secondary antibodies**

Secondary antibodies were used as indicated in Table 3.2 in the appendix to Chapter 3, again diluted to the indicated concentration in Dako antibody diluent. Slides were wiped to circumscribe the tissue section and 80-100 ul of secondary antibody solution was applied. The tissue was incubated for 60-90 minutes at 4ºC without applying Parafilm, and then again washed 3 times for 5 minutes each in changes of PBS with 0.1% Triton X-100.

**Tertiary chromogens**

If the secondary used was dependent on a peroxidase reaction for chromogen development, a tertiary linkage was used containing Horseradish Peroxidase bound to Streptavidin (ExtrAvidin-Peroxidase™, Sigma-Aldrich) used at a dilution of 1:100 in antibody diluent and applied for 20 minutes. The slides were washed 3 times with PBS + 0.1% Triton.

Two types of chromogen were used. 3,3'-Diaminobenzidine (DAB) solution was prepared from 10 mg tablets (Sigma-Alrich) made up in 15 ml of 0.05% Tris buffer to which 15 ul of 30% hydrogen peroxide was added immediately prior to use. 3-Amino-9-ethylcarbazole (AEC) was used as directed from a kit obtained from Sigma-Aldrich. The kit contains 3 mL of concentrated acetate buffer, 3-amino-9-ethylcarbazole (AEC) chromogen and hydrogen peroxide dropper bottles for easy dispensing. The solution is made up in 4 ml deionized water by adding 2 drops Acetate Buffer (Vial 1), 1 drop AEC Chromogen (Vial 2), and 1 drop 3% hydrogen peroxide (Vial 3). A preference was for the use of AEC, given concerns of DAB being carcinogenic. DAB or AEC chromogen solutions were applied to the tissue sections for time intervals of
2 minutes minimum to 15 minutes maximum to develop the colour signal, looking at the sections every 2-3 minutes under the light microscope to directly assess the reaction time. The solutions were then cleared under running tap water for 5 minutes.

**Mounting**

If the secondary antibodies were linked to fluorochromes, care was taken to minimize exposure of the slides to direct light. The tissue was directly mounted in specialized fluorescent mounting media (Dako) to preserve the antibody fluorescence and to limit tissue auto-fluorescence. If DAB was used, then the tissue needed to be dehydrated again prior to coverslip mounting. Dehydration was done with increasing concentrations of ethyl alcohol rinsing the sections gently for 5 minutes in 50%, 80%, 90%, then 2 changes of absolute alcohol prior to 2 changes of xylol. Some section slides were lightly counterstained with hematoxylin, with a minimal incubation time of 20-30 seconds followed by rinsing under tap water, dehydration through alcohol and xylol, and mounting under DPX. For AEC sections, it was not necessary to dehydrate the sections and these were mounted directly with aqueous mounting media (Aquatex™, Merck), and dried overnight at room temperature.

**Double immunostaining**

Where feasible, immunostaining was done using 2 separate primary antibodies on the same section, provided they were of separate Ig class to enable two different secondary antibodies to distinguish between them. When double stains were done, both antibodies were diluted into the same diluent buffer to the optimal concentration. No changes were made in the concentrations of either primary or secondary antibodies over what would have been used for a single stain. On occasion the nuclear stain DAPI (Invitrogen) was added to the secondary diluent at a concentration of 300 µM, and visualized in the blue 460 nm range.

**Immunohistochemistry controls**

For each antibody application, appropriate controls were developed. Positive control tissue, including uninjured rat spinal cord and rat spleen, were used to verify antibody specificity. Once verified, the optimal primary antibody
dilution was determined on the test tissue through a series of primary antibody dilutions, usually 1:20, 1:50, 1:100 and 1:200 assessed in parallel. Secondary only controls were done on the same test tissue as was being analysed at the time in full. The purpose of secondary only controls was to verify there was no non-specific signal from the tissue, as either autofluorescence or from secondary antibody binding.

A number of other controls to optimize the sequence of the staining protocol were done. Optimization steps included:

- reducing the percentage of hydrogen peroxide used in the peroxidase blocking step, from 3 % to 1.5% to see if this resulted in less tissue damage,
- varying the sequence of the peroxidase blocking step, from being before the antigen retrieval step to being after the retrieval step,
- varying the concentration of secondary antibody (from 1:20, 1:50 and 1:100),
- varying the concentration of Sigma’s HRP-Peroxidase (from 1:20, 1:50 and 1:100) to reduce background staining,
- assessing the tissue for endogenous peroxidase activity by not using any primary, secondary or HRP-peroxidase, and simply adding the chromogen-hydrogen peroxide reaction solution to the tissue.

3.2.4 Light and Fluorescent Microscopy

All microscopy images were taken at NUI Galway in the Department of Anatomy by myself, Eva Sweeney and Lisa Kinnavane.

Light microscopy

Images of chromogen stained channels were taken with the Leica DM1000 Upright Fluorescence Microscope. Objectives of 2.5x, 5x, 10x, 20x, 40x, 100x
(Oil emersion) were used and the images captured after white balancing with a Leica DFC300FX Camera, using the Leica Application Software.

**Fluorescence microscopy**

For all fluorescent imaging, except those involving a Cy-5 secondary, the department’s Structured Light Microscope Olympus IX81 Inverted Microscope was used. Its objectives included 4x, 10x, 20x, 40x, 60x Oil Emersion. Images were captured after setting gain and contrast appropriately using a Hamamatsu Orca ER Camera, and Volocity (Improvision) Software. For Z-Stacking of images, the images were compiled from an 8 micron thickness with slices of 0.5 microns moving from the bottom of the section upwards. Here, an Optigrid structured light device was also used.

Images of the eGFP-MSC group combined cellular fluorescence in the FITC wavelength with TRITC and Cy-5 emission from secondary antibodies. The Anatomy Department’s Olympus IX81 Inverted Microscope with a Yokogawa CSU-X1 Spinning Disk Unit was used. All imaging with this microscope were taken by Eva Sweeney given her technical expertise. Objective lenses included a 10x, 20x, 40x Oil, 40x Long Working Distance, 60x Water and a 60x Oil Emersion lense. The images were captured using an Andor iXon EM Camera, using Andor IQ Software.

**3.2.5 Scaffold and Channel Image Acquisition**

A structured scheme was developed to acquire images of scaffold channels in a coordinated manner. This process was necessary given the large numbers of images generated and enabled comparison across animal groups at specific scaffold intervals. It also offered the potential to track the structure of individual channels down the scaffold length.
Figure 3.1: Schematic for imaging scaffold channels. For each level of the scaffold, an overview image was taken of the section on the slide that best preserved the scaffold architecture. Channels were counted from 1-7 in a clockwise fashion from the uppermost channel, and with the central always being designated as 7. It was often possible to trace a particular channel down the length of the scaffold by noting landmarks, here illustrated as a torn channel interface between #6 and #7, an apical nerve root, and the overall (elongated) shape of the section. Each channel was then photographed individually in a series for subsequent image analysis.

Slides at each one quarter interval of each scaffold (n=18 per animal group) were stained in parallel. Once cover-slipped and dried, the slides were viewed under microscopy and imaged. For each slide, the optimal scaffold section was chosen under a low power field (4x without use of the condenser) as that which had the most channels accurately preserved within the overall architecture. This section was labelled with a marker pen, and was then imaged in full to provide orientation for the subsequent channel images. Each channel then was imaged individually under 10x or 20x power, in a defined sequence: the topmost
channel was designated as channel 1, and images were then taken in a clockwise fashion (channels 1-6); the central channel was imaged last, as being channel 7.

For each antibody stain of interest, there were 3 scaffold levels imaged within the 6 per animal group, or 18 sections per group, with 8 total images taken per section including the overview image. This yielded 144 images per group for analysis as a maximum value. Often not all 7 channels within a section were suitable for imaging, as they were distorted by tissue processing or the channel had been surrounded by the ingrowth of tissue within the scaffold. However, an analysis of the numbers of channel observations, and the means by which channels became distorted, was made possible by this rigorous scheme.

3.2.6 Quantifying the Structural and Cellular Composition of Channels

Image analysis of scaffold channels was done with the expert assistance of Lisa Kinnavane from the Department of Anatomy, NUI Galway, using the NIH software, ImageJ. The software is freeware, and available for download at [http://rsbweb.nih.gov/ij/download.html](http://rsbweb.nih.gov/ij/download.html)

**Area measurements in scaffold channels**

Slides stained for neurofilament quantification were also used for analysis of total channel area and the contributions of the central core and peripheral layers. The area of each channel was calculated by capturing an image of a graticule on the same microscope, at the same magnification as the original images. The image of the graticule was opened in ImageJ and the length, in pixels, of a set division on the graticule was noted. This length was calibrated into the software to calculate the length and thus the area that each pixel in the image represented. The area per pixel was then multiplied by the number of pixels in the region of interest to give the image area in $\mu m^2$. Areas were determined for each channel. Where the core structure could be clearly distinguished from the circumferential periphery of tissue, the area of the core was similarly determined. The area of the periphery was calculated by subtracting the core from total channel area.
Quantification of scaffold cell composition

Scaffold tissue sections for each animal group were double stained with primary antibodies to Glial Fibrillary Acid Protein (GFAP) (Rabbit anti-rat, 1:100, Dako) and the S-100 antigen (mouse anti-rat, 1:300, Biogenex San Ramon CA USA). For Matrigel and SC sections, GFAP was linked to an AlexaFluor™ 546 anti-rabbit IgG secondary antibody (1:100, Invitrogen Molecular Probes, Dun Laoghaire Ireland), which is seen as bright orange in the TRITC wavelength. S-100 primary was bound by an anti-mouse IgG secondary linked to a FITC label (1:100, Millipore Chemicon, Carrigtwohill Ireland). For MSC sections, innate eGFP expressed by the cells was seen in the FITC wavelength, GFAP in the TRITC, and the S-100 was visualized using a Cy-5 linked anti-mouse IgG secondary (Millipore Chemicon), seen in the Far Red spectrum. Additional primary antibody staining was done on separate sections for the chondroitin sulphate proteoglycan Neuroglycan-2 (NG-2) (1:100, mouse anti-human, Millipore Chemicon). NG-2 distribution was visualized using a goat anti-mouse IgG secondary (Sigma) labelled for TRITC fluorescence. (NG-2 staining was combined with antibodies against Collagen IV for vessel analysis.) The intermediate filament Vimentin was localized using a mouse anti-rat primary (1:100, BD Pharmigen, Oxford UK) and either the anti mouse IgG FITC or Cy-5 secondary antibodies. (Vimentin staining was combined with IBA1 antibodies analysis as part of the immune cell infiltrate analysis.)

Immunofluorescence image analysis

Following the acquisition of images from each channel in each fluorescence wavelength, the images were analyzed using Image J to determine the proportion of area stained per channel area. For double stained, DAPI fluorescence images, the colour channels were split into separate images, each containing only the information of red, green or blue respectively. Single stained images were left in their original emission colour. All images were then converted to greyscale and the colours were inverted to allow for easier identification of positively stained tissue. An intensity threshold level for each colour was then set for each stain, to include the area that were clearly stained but to exclude non specific staining, artefact, or areas of the image relating to the channel structure.
This threshold level was kept constant for all images analysed for each stain; however, thresholds varied between stains.

When the threshold was applied, the image was then converted to a 1 bit black and white image where the pixels with an intensity level between the upper and lower threshold limit turn black and all remaining pixels turn white. A region of interest was set to include only the channel tissue. The percentage density of staining was calculated as being percentage of black coloured pixels to the number of pixels in the entire region of interest.

Statistical analysis was done as a comparison of means with Kruskal-Wallis ANOVA and a Dunn’s multiple comparison of means post test.

3.2.7 Quantification of the Inflammatory Infiltrate within Channels

The degree of chronic inflammation within the scaffold channels was characterized by immunohistochemistry and image analysis to determine the infiltrate proportion within the channel for each animal group.

Leukocyte, T-cell and Macrophage Identification

Primary antibodies to the leukocyte common antigen (CD45R (mouse anti-rat 1:50) (BD Pharmingen) were used with a biotinylated rat absorbed anti-mouse secondary antibody (Dako); the reaction was developed with the AEC chromogen. Two primary antibodies to macrophages were used, the first being CD11b/c (mouse anti-rat, 1:200), with a biotinylated secondary, and the second being Lba1, the Ionized calcium binding adaptor molecule 1 present on rat microglia and macrophages, (rabbit anti-rat, 1:100) (Wako Chemicals GmbH, Neuss Germany). The latter was used in conjunction with a goat anti-rabbit AlexaFluor546 (bright orange) secondary (Molecular Probes, 1:100) as part of a double stain procedure with vimentin. The proportion of T-Cells was quantitated using a primary antibody against CD3 (mouse anti rat IgM, 1:20 (Serotec) along with goat anti-mouse IgM-FITC conjugate, 1:100 (Sigma-Aldrich) for the Matrigel and Schwann cell groups, and a Cy-5 (Far Red) conjugated anti-IgM secondary for the MSC groups.
Chromogen immunohistochemistry image analysis

In slides developed with peroxidase reactions and DAB or AEC chromogens, the images similarly were converted to greyscale using the ImageJ software. A threshold level was set for the stain and applied, converting the image to a 1 bit, black and white image. A region of interest was then set to include only the channel tissue. The percentage density of staining was calculated as being the proportion of black pixels to the total number of pixels in the region of interest.

Statistical analysis was done as a comparison of means with Kruskal-Wallis ANOVA and a Dunn’s multiple comparison of means post test.

3.2.8 Axonal Profile Counting and Data Analysis.

Neurofilament staining (mouse anti-rat monoclonal antibody 1:50, with a biotinylated secondary antibody (Dako North America, Carpinteria CA)) was used in serial transverse sections to identify axon fibres within the channels of the scaffold. DAB was used as the chromogen for all neurofilament staining. The technique for axon counting has become standard in our laboratory (Krych et al., 2009; Olson et al., 2009).

Axon counting criteria

Select criteria for counting neurofilament stained axons were developed and employed. These included:

1. Selecting best section on the slide and identifying that section with a marker pen.
2. Sketching the section and labelling the channels on a separate paper pad.
3. Identifying and noting on the drawing any positive neurofilament staining other than that which was within the channels, to verify that the stain is working. This was especially important for negative channels. Also it was important to note heavily stained macrophage areas, as the DAB chromogen was also taken up by these cells.
4. Positive neurofilament fibres were counted using 20X and 40X objectives, primarily using the 40X objective. The microscope employed was located at the Mayo Clinic, a Ziess Axio Imager Z1 microscope with a mounted Ziess Axiocam
and KS400 software. The 100X objective was used to isolate the individual fibres of positively stained axonal clusters. As a rule, if the positive fibres appeared in the 100X objective but were not identified with the 40X objective, they were not counted, as it should be possible to visualize all positively stained axons under the 40X objective.

5. Channels were identified by exterior laminar wrapping and central core of likely positive neurofilament staining and ‘transverse’ cell appearance. Some caution needed to be taken when evaluating areas with debris, and heaped up exterior edges that may be have been mistaken for channels.

6. Brown translucent material was commonly found in the core of some channels, which probably represented Matrigel, but positive fibres in both transverse & longitudinal sections could be found within this material. Positive fibres within the Matrigel were smaller, more focused and more abundant:

7. If a channel was missing or folded in the selected section, it was sometimes possible to move to an earlier or later section (8 µm difference) and count the positively staining NF fibers in that channel.

8. If one was in doubt, the stained area was not included into the total count.

**Axon counting**

Stained axons in each identified channel were counted using 40x and 100x objective lenses at defined scaffold levels of 1/4, 1/2, and 3/4 the length distally from the rostral and caudal scaffold-cord interfaces. The interfaces were defined as the first and last transverse section in which polymer could be seen and the quarter length sections calculated accordingly from the total number of slides comprising the scaffold. Each level of the scaffold was counted by a blinded observer in triplicate, and the mean count was treated as an independent observation, given that axons can arise from either rostral or caudal cord. For each animal, the total axon number within the scaffold channels was counted as being the sum of counts across each level, and was treated as an independent observation between animal groups. The Kruskal–Wallis test (nonparametric ANOVA) was used determine if there was a significant difference in median axonal counts between the three animal groups.
3.2.9 Unbiased Stereology for Blood Vessel Quantification in Scaffolds

To analyze the blood vessel structure within the scaffolds, 8 micron scaffold sections were stained with primary antibody to Collagen IV (1:300, polyclonal rabbit IgG, Abcam, Cambridge England) and visualized with either a FITC goat ant-rabbit IgG secondary (1:200, Abcam) in the Matrigel and SC groups, or Cy-5 Far Red secondary in the case of the MSC group. Images were acquired at 20 x magnification using the structured light microscope for Matrigel and SC, and the spinning disk confocal microscope for the far red images. An unbiased stereology approach (Garcia et al., 2007; Howard, 2010) was used to make estimates of volume fraction (Vv), length volume/density (Lv) and surface volume/density (Sv) of blood vessels within each channel at quarter length intervals through the scaffold.

**Volume fraction (Vv)**

Volume fraction represents the proportion of each unit volume in the reference channel space that is occupied by a vessel structure. A simple point grid (Figure 3.2) was photocopied onto a transparency and overlaid onto the channel image field in a random orientation. The number points hitting a vessel feature was counted. This total was then divided by the number of encircled points hitting the channel surface. The count of encircled points was then multiplied by 9, as the ratio of encircled to normal points is 1:9, yielding the total number of points in the reference space.

\[ V_v = \frac{\text{Volume of vessel feature}}{\text{Volume of reference space}} \]
Figure 3.2: A simple point grid to estimate volume fraction. The total number of points overlaying vessel structures was counted. The reference volume is calculated by counting the encircled points and multiplying by nine.

It is not correct to average the individual estimates of volume fraction over all the fields as a biased estimate will be obtained. The volume fraction is calculated as the total sum of feature points divided by the total sum of reference points,

$$Vv(Y, \text{ref}) = \frac{\sum_{i=1}^{m} P(Y)i}{\sum_{i=1}^{m} P(\text{ref})i}$$

where $Y$ is the vessel feature, $\text{ref}$ is the channel surface, $m$ is the number of fields analyzed, and $i$ is the number of point intersections.
**Length density ($L_v$)**

The combined length of vessel structures embedded within a channel section, or the total length of vessel per unit volume, is known as the length density.

$$L_v(Y, \text{ref}) = \frac{\text{Length of vessel feature}}{\text{Volume of reference space}}$$

This estimate is related to the number of vessel structures multiplied by 2,

$$L_v = 2 \cdot Q_A$$

where $Q_A$ is the number of profiles per unit area of the test probe.

In order to obtain an unbiased count of the vessel number, a grid of counting frames was used. The frame (Figure 3.3) consists of a solid “forbidden line” which extends above and below the field of view (to infinity), and a dashed “acceptance line.” The area ($a$) of the counting frame ($\Delta x \times \Delta y$ units squared) is known as it relates to the final magnification of the image. The area of the counting frame is associated with a central point, used to measure the reference volume. A vessel was counted if it fell either fully within the frame area or lay partially outside and overlapped with the dashed acceptance line. Any vessel that overlapped the solid forbidden line in any way, or fell outside of the frame area (the “guarded area”) was not counted.
A photocopy of a counting frame grid, comprising 15 counting frames, was made onto a transparency and the grid laid over the channel image in a random orientation. The length density was calculated as the ratio of the sum of grid-vessel intersections (i) over the sum of points falling in the reference channel space over a given field number (n),

$$L_v(Y,\text{ref}) = 2 \cdot \frac{\sum_{i=1}^{n} Q_i}{a/f \cdot \sum_{i=1}^{n} P_i}$$

where $Q$ is the number of vessel profiles correctly sampled by the frame, $P$ is the number of frame-associated points, and $a/f$ is the area of the frame at the final magnification (3,600 µm$^2$).
Length density is an important physiology parameter as it applies inversely to the radial diffusion distance, a robust indication of a cylindrical zone of diffusion around the vessel wall (Dockery and Fraher, 2007). This parameter was calculated from \( L_v \) estimates according to the equation:

\[
\rho(\text{diff}) = \frac{1}{\sqrt{\pi} \cdot L_v}
\]

**Surface density \((S_v)\)**

The surface area of blood vessel features per volume of the reference space is known as the surface density of the vessels.

\[
S_v(Y, \text{ref}) = \frac{\text{Area of vessel interface } Y}{\text{Volume of the reference space}}
\]

In this estimate, a series of linear test probes associated with a reference point was used (Figure 3.4). The relationship between the number of intersections between the test line and vessel surface \((I_L)\), and the vessel surface area per unit volume, is given by the equation:

\[
S_v = 2 \cdot I_L
\]

For isotropic uniform random sections (IUR), the \( S_v \) is calculated as twice the sum of number the line-vessel intersections in inverse proportion to the sum of points striking the channel surface over a given field number \((n)\), provided the length of the line associated with the reference point \((l/p)\) is known at the final magnification, here 60 \( \mu \text{m} \).

\[
S_v(Y, \text{ref}) = \frac{2 \cdot \sum_{i=1}^{n} I_i}{l/p \cdot \sum_{i=1}^{n} P_i}
\]
Figure 3.4: A line grid used for surface density estimation.

Mean vessel diameter and cross-sectional area.

Two other key physiologic parameters are derived from proportions of volume fraction, length density and surface density (Dockery and Fraher, 2007). The mean vessel diameter was be calculated from the ratio of surface to length density according to the equation:

$$
\bar{d} = \frac{Sv}{Lv\pi}
$$

Vessel diameter calculation has critical implications for blood flow rate through a section of the channel, as the flow rate is proportional to the fourth power of vessel radius.
Similarly, the mean cross-sectional area is obtained combining all three stereologic estimates and derived from the diameter calculation above, as being

\[ \bar{a} = \frac{V_v \pi d^2}{L_v 4} \]

**Total volumes as applied to scaffolds in situ.**

Because each of the stereologic estimates represents proportional fractions, anatomical estimates of value for scaffolds *in situ* can be made if the reference volumes are known. For example in the case of volume fraction, (and the same equation holds true for length and surface density), the relationship of total volume is determined by

\[ V(Y) = V(\text{ref}) \cdot V_v(Y, \text{ref}) \]

For each of the estimates, the average channel volume \( V(\text{ref}) \) can be obtained by multiplying the mean channel area at a corresponding scaffold and its quarter interval by the approximate thickness of the tissue section, 8 microns.

**Correlations between axon number and vessel stereology estimates.**

To determine if a significant relationship existed between the various vessel stereologic estimates and axon number, axon and vessel data was obtained and verified as being from the same animal, and from the same quarter length through the scaffold. Data pairs were then plotted and statistical correlation was calculated using GraphPad 5 software, assuming a non-parametric correlation yielding Spearman r coefficients and P-values by two-tailed analysis.
3.3 Results

3.3.1 Integrity of Scaffold Channel Architecture Following Tissue Sectioning

As seen in Chapter 2, the majority of scaffolds were well aligned with the surrounding cord. The scaffolds were fully integrated into the native cord tissue by means of a fibrous capsule surrounding it, by scarring at each interface with the cord, and by the extension of cord tissue through the scaffold channels as ‘tissue cables.’ Scaffolds embedded in paraffin were sectioned at 8 µm lengths. During slide processing, particularly during deparaffinising and graded alcohol rehydration steps, the hydrogel polymer was dissolved free from the section. At low magnification, the overall architecture of the scaffold in maintaining a 6+1 channel orientation was typically well preserved following sectioning (Figure 3.5). What is notable in these images is the accuracy of the spatial orientation of channels. The multilayered structure of the capsule surrounding the scaffold shows is also apparent. A thin layer of dense fibrotic, acellu lar tissue, has formed around the circumference of the scaffold. This rim is itself the underside of a discrete cellular layer separating the implant from tissue innate to the spinal cord, in which nerve roots are commonly found.

When the architecture became disrupted, there were several ways seen to limit the number of channels that could be analysed at a particular level. Figure 3.6 depicts such changes to the structure, including:

- Residual hydrogel being present on the section and obscuring the channel (A, B)
- Channels being absorbed into a proliferation of tissue, typically seen at the ¼ or ¾ length sections near the cord interfaces (C, D)
- Channels growing into each other (E)
- Channels disrupted by formation a large cyst, usually within the central core of the scaffold (F)
- Channels being transposed outside of the scaffold boundary during sectioning or on top of another portion of the scaffold (G, H)
- Channels being folded upon themselves by sectioning (F, H)
Figure 3.5: Seen at low 4x magnification, the architecture of a Schwann cell (A) and an MSC (B) animal scaffold has been maintained after 4 weeks post implantation, and following tissue processing and sectioning.
Figure 3.6: The architecture of scaffold was seen occasionally to become disrupted by either tissue growth, or tissue processing artefact. Bar = 50 μm
Channel numbers

To assess what impact these factors may have had on decreasing the numbers of channels available for analysis, four groups of tissue slides representative of the histologic techniques employed were selected across each of the three animal groups, including H&E, GFAP and Iba1 (fluorescent), and CD45 (AEC) sections. The mean number of channel observations at quarter interval levels within each group was calculated using 72 Matrigel and Schwann cell scaffold sections, and 69 eGFP-MSC sections (Figure 3.7). In panel (A), there was a trend within two groups to have less channels available for analysis at the rostral and caudal ends of the scaffold at its cord interface. This was seen in the Matrigel group at the ¾ level, in the eGFP-MSC group at the ¼ level. In the Schwann cell group a reduction in the channel number was seen at the ½ level. There was no statistical significance between these means; however the trend is consistent with tissue ingrowth observed at the ¼ and ¾ levels, and cystic cavities occasionally seen within the Schwann cell scaffolds at their midpoint.

In Panel (B) when all sections at either the ¼, ½ or ¾ scaffold level were included in the analysis regardless of their animal group, the mean number of channel observations was highly consistent. The mean number of ¼ level observations was 5.49 +/- 0.21 channels, 5.52 +/- 0.21 channels at the ½ level and 5.28 +/- 0.23 channels at the ¾ level.

Panel (C) depicts a histogram representing the frequency with which a given number of channels was intact in the section, with the range being from none with complete disruption of the scaffold architecture, to the maximum seven in a fully intact scaffold. All seven full channels were fully intact with the highest frequency in all animals groups, available for analysis in 36.62%, 42.24% and 39.44% of the scaffold sections at the ¼, ½ and ¾ scaffold level. Five or more channels were intact in 78.87%, 77.46% and 69.01% of scaffold ¼, ½, and ¾ levels, while 3 channels or less were seen in 11.27%, 18.21% and 15.49% of respective quarter scaffold intervals.
**Figure 3.7:** The numbers of channels intact and available for analysis did not statistically vary at a given scaffold level, either within a particular animal group (A) or at a given level across all animal groups (B). Most frequently, all 7 channels were available for analysis, seen in a range between 36 and 42% of scaffold sections (C).

**Comparisons of channel area**

The OPF+ hydrogel scaffold is a highly dynamic structure, on the basis of both its charge and its state of hydration. The cross sectional area of a given channel area had been estimated to be on the order of 158,960 $\mu m^2$, given a channel diameter of 450 $\mu m$. To determine the effect of tissue fixation and sectioning on channel size, channel area in each of the animal groups, and at each scaffold quarter interval was measured. The total number of individual channel observations was 94 for Matrigel, 112 for MSC and 55 for SC channels down their length. Channel cross sectional area was calculated using image analysis software (NIH ImageJ). After imaging a graticule at the same magnification as the original image, the number of pixels occupied by the channel area was converted to surface area.

**Figure 3.8:** The cross sectional area of scaffold channels after fixation. (A) Mean channel area as compared across the 3 animal groups. (B) Mean area as measured at each scaffold quarter interval.
The mean cross sectional area for Matrigel scaffold channels (120,990 +/- 1806 \( \mu \text{m}^2 \)) was significantly higher than the eGFP-MSC group (106,691 +/- 1677 \( \mu \text{m}^2 \)) \((p<0.001)\). The eGFP-MSC channel area itself was significantly smaller than the Schwann cell channel area, which averaged to be 114,264 +/- 2363 \( \mu \text{m}^2 \) \((p<0.01)\). There were no significant differences between Matrigel and Schwann cell channel areas. With fixation, channels were reduced to 77% of their pre-implantation size in the Matrigel group, to 71% in the SC group, and to 67% in the MSC group. The degree to which the eGFP-MSC channels shrank more may be attributable to the looser, highly vascular type of tissue which developed within the channel. The standard error of the mean in each group was less than 2% of the mean value, indicating a high degree of reproducibility and preservation of the scaffold structure. Equally, when the channel size was analysed at each quarter level of the scaffolds, the only significant change within an animal grouping was seen between the quarter and half level of the Schwann cell scaffold (n=20 and n=17 respectively).

### 3.3.2 Tissue Architecture of Matrigel, Schwann cell and MSC Scaffold Channels

**Evaluation with haematoxylin and eosin, Masson’s Trichrome**

To assess the cellular architecture of scaffold channels in each of the animal groups, sections at quarter length intervals were stained with Hematoxylin and Eosin. A limited number of sections with Masson’s Trichrome for light microscopy. The aim was to provide a general histopathologic view of the tissue that had form in association with cell-loading after 4 weeks *in vivo*, and to comment upon any differences observed between the groups.

Figure 3.9 shows a representative channel from each of the Matrigel (A), Schwann cell (B) and eGFP-MSC (C) groups stained with H&E, with an additional eGFP-MSC channel stained with Masson’s trichrome (D). All channels are seen to highly cellular and without areas of gross tissue necrosis. In the Matrigel channel, discrete regions of different tissue types are immediately in evidence. A central, circular core of loose tissue is circumscribed by more dense, laminar tissue extending to the channel wall. The impression is of a central core
and of a peripheral boundary of different tissue types. There is some edge effect of
the stain. However the density of nuclei staining increases towards the periphery
and some areas of dense nuclear aggregates are seen where the tissue would have
been in contact with the OPF polymer are suggestive of foreign body reaction.
There are a number open spaces that are sharply defined, and are either confined
to the core area or abut the boundary with the denser peripheral zone. Some of
open spaces have clear endothelia, indicative of vessel formation and other areas
may represent walled cysts or cavities. A macrophage and small endothelialized
vessel are indicated with arrows.

In the Schwann cell channel, again the core area is present, here offset to
the left lower edge of the channel. The core has relatively a more uniform
cellularity and cell density than the Matrigel channel; the open spaces appear
smaller and perhaps more abundant. The peripheral tissue is densely cellular out
to the edge, and here looks almost columnar in appearance. The arrows show an
area of sharply linear demarcation sometimes seen between tissue types within the
channel.

In the eGFP-MSC channel, the most striking difference is the number of
large endothelia-lined vessels occupying the core. The core itself is hypocellular,
and again is clearly demarcated from the circumferential tissue, although here the
core extends nearly to the channel edge with a thin peripheral layer. The MSC
channel slides were reviewed by Joseph Parisi M.D., a Neuropathology and
Anatomic Pathology consultant at the Mayo Clinic. His opinion was that the
tissue type was remarkably similar to inflammatory granulation tissue. In (D)
another MSC channel was stained with Masson’s Trichrome to better define the
vascular endothelia, and several of the smaller vessels in the core can be seen to
contain erythrocytes. Also seen in (D) are multinucleated aggregates darkly
staining on both sided of the top of the channel.

**Figure 3.9:** (Opposite) Centralized core and peripheral tissue compartments are
seen in representative sections tissue sections from Matrigel (A), Schwann cell
(B). The periphery is more dense and contains multinucleated aggregate the
channel interface. The core is more open, with variable cellularity across the
groups, and contains vessel structures and cystic spaces (continued opposite).
(Figure 3.9, continued) Arrows in (A) highlight a macrophage, and a small endothelialized vessel. In (B) the arrows show a sharp demarcation of tissue types at the boundary between core and peripheral tissue types.
Figure 3.9 C and D: Prominent vaculature is indicated with arrows in D, in this Masson’s Trichrome section. (Bar: 20 µm in A-C, 10 µm in D). and eGFP-MSC channels (C) stained with hematoxylin and eosin.

Overall for each group, the pattern is of chronic inflammatory infiltrate with a peripheral foreign body reaction along the outer edge. In each case a core tissue type can be histologically distinguished from a circumferential type. The MSC group is strikingly different in the degree of hypocellularity in the core and the extent of large vessel formation. The increasing cellular density towards the edges suggests that the expansion of tissue formation in vivo may have been from the core outwards as the tissue has become compressed against polymer edge.
**Structural analysis using eosin autofluorescence**

Using H&E stained sections, it was also possible to analyse the structure of channels with reference to their matrix and fibrous content. Eosin will fluoresce at FITC 490 nm excitation and to a lesser degree at TRITC 545 nm excitation laser wavelengths, to illuminate the cellular components to which it binds. Eosin is an anionic dye, binding acidophilic structures, including elastic fibres, basal lamina, mitochondrial aggregates, chromatin and mitotic spindles (Jakubovsky et al., 2002). For our purposes, the fluorescent images were used to assess the overall cellular architecture of the scaffold channels in relation to vessel formation and the concentration density of elastic tissue.

Figure 3.10 shows three representative channels from the Matrigel group. Consistent with what was noted in the H&E brightfield imaging, different tissue types comprising a channel core and a circumferential periphery are strikingly apparent. There is a ring of fibrous tissue that is brightly fluorescent, separating the channel core tissue from the periphery. The channel core is less densely fibrous, and accommodates several small vessel structures whose basal lamina is seen. There is a tight layering, or laminar structuring of the fibrous tissue encircling the channel core, and this structure transitions to an outer ring of tissue that is less dense in elastic fibres at the outer edges of the Matrigel channels. Nuclei appear as small open spaces throughout the sections. Multinuclear aggregates are again seen at the channel interfaces with the polymer.
Figure 3.10: Matrigel channels are visualized with eosin fluorescence in (A-C). The channels are now seen to have a 3-tiered structure. A central core that has relatively less elastic tissue density contains the space into which vessels can extend. The core is ‘walled off’ by a dense fibrillar banding, before the structure transitions to its interface with the channel polymer. Here features of multinucleated aggregates are seen (arrow); the area is relatively devoid of vascular structure. Bar = 50 μm

Three representative images of Schwann cell filled channels are shown on the next page, Figure 3.11. Consistent with the H&E brightfield images, the structure is more homogenous in its tissue density and here in its fluorescence than the Matrigel channels. A central core can also be seen, as in (B) where the transition between core and periphery is clearly demarcated and in (C) where the structure more resembles a Matrigel channel regarding a tight banding of fibrous structures that separate it from the peripheral zone. Within the core of Schwann cell channels, the structure is quite different to Matrigel in that there are characteristic stellate patterns of staining around open centres, which has been highlighted in the insert of panel (A). These patterns may represent small blood vessel beds, nuclei surrounded by stellate cytoskeleton, reactive astrocyte cytoskeleton, or perhaps may even represent axonal tracts surrounded by supportive cells, such as Schwann cells.
Figure 3.12

A

B

C
**Figure 3.11:** Schwann cell channels (A-C) have a relatively more homogeneous core characterized by stellate structures surrounding small lumenal space, magnified for detail in the insert. These structures may represent the lumen of capillary beds, axonal tracts surrounded by Schwann cells, astrocyte cytoskeleton or spaces representing cell nuclei. The core and peripheral compartments are sharply demarcated by a wall type structure (B) of fibrillary banding.

*Bar = 50 µm*

In Figure 3.12 A-E, fluorescent images of representative eGFP-MSC channels are shown. The distribution of tissue that has a high elastic content is primarily in relation to the prominent vasculature in the core, which is essentially comprised of walled vessels and open spaces whose diameter is far greater that that seen in the other groups. The outer periphery appears to have a similar structure to the other groups, and as in (C) there is similar, laminar banding that separates the core from the periphery. No stellate structures are seen. In E, composite Z-stacked imaging of the 8 micron section demonstrates the channel architecture in 3 dimensions, showing depth to vessel lengths (*). There is layering that is occurring with the elastic tissue, forming peaks and troughs that are almost in concentric rings around the vessel-filled core. In the bottom right corner, the fibrous banding of the scaffold capsule can also be seen, phase of the project.
**Figure 3.12**

(A) (B) (C) (D) (E)
**Figure 3.12:** eGFP-MSC channels with eosin fluorescence detailing various examples (A-D) of the prominent, large calibre vessels and open cystic spaces in the core. Here the vasculature and its support tissue comprise the substance of the core. In E, Z-stacking of 20 images at 0.5 $\mu$m intervals provides structural depth to the vessels through the channel. Bar = 50 $\mu$m A-D, 20 $\mu$m E.

As a measure of the elastic tissue content for each scaffold channel type, the area of fluorescence was measured in proportion to the total area of a channel surface was measured. As anticipated given the degree of open space within the MSC channels, be it vessel, cyst or cavity, the MSC group had significantly less fluorescing tissue per channel area (13.21 +/- 0.66 %, n=90 channels) than both the Schwann cell (17.61 +/- 0.73 %, n=89) and Matrigel groups (22.21 +/- 0.74 %, n=107, p<0.0001). The proportion was low despite the some contribute of the MSC cells themselves to the area of fluorescence. Matrigel had significantly more density of fluorescent tissue that the Schwann cell group (p<0.0001). No significant differences were seen between quarter length intervals within a particular group.

**Figure 3.13:** The proportion of channel area fluorescing with eosin stain as an indication of the elastic tissue content of channel. (A) A comparison of channels at all levels across the three groups. (B) An analysis at quarter length intervals down the scaffold within each group. No significant differences were seen within a particular animal type.
3.3.3 Identification of the Cellular Components in Scaffold Channels.

With the observation that scaffold channels were compartmentalized by means of a core area that was structurally separated from a peripheral zone, we set about to identify what cell types might be contributing to each compartment.

\textit{eGFP-MSC fluorescence}

In eGFP-MSC animal group, MSC cells were seen to be present by eGFP fluorescence within scaffold channels at 4 weeks. The cells localized to the central core, in proximity and often closely associated to vessel structures.
**Figure 3.14:** eGFP-MSCs are present in the core compartment of scaffold channels at 4 weeks post implantation. The rounded cells are seen in close association with adjacent vessel walls, in a. Two example of this distinct perivascular distribution are shown in (A) and (B), where the arrow indicates the cells may be actually integral to the vessel structure. Bar=10 µm.

**Glial Fibrillary Acid Protein Immunostaining**

Channel sections were immunostained for Glial Fibrillary Acidic Protein (GFAP), an astrocyte marker identifying mature astrocytes, and which is upregulated during reactive astrocytosis and CNS injury. GFAP positive cells were seen to uniformly line the channel periphery, separated from central core area which had a different pattern of staining isolated to individual cells or clusters. In the periphery, the GFAP cell structure was tightly packed, and became more so towards the exterior of the channel where the tissue interfaces with the
polymer. The staining pattern was initially noted using a DAB chromagen but was much more vividly seen in fluorescent imaging.

![Image](image_url)

**Figure 3.15:** GFAP-positive astrocytes were seen to localize to the channel periphery in a concentric pattern in a Schwann cell channel. In (A), primary antibody to GFAP was bound by a biotinylated secondary and the DAB chromogen, while in (B) the secondary has a TRITC label. In both methods, the staining intensified towards the outer circumference, which is in part an edge effect at the outermost periphery, but also an indication of increased cellular density as the cells packed against the inner channel wall. An area of central clearing is noted, with scattered cells staining positive in the channel core. In this instance, both scaffold were from Matrigel control animals. Bar = 20 µm.

The pattern of GFAP staining was evident in each of the animal groups. Figure 3.16 details a Z-stacked image of an eGFP-MSC channel showing perivascular MSC location as well as the peripheral rim of GFAP positive tissue.
Figure 3.16: GFAP staining within an MSC loaded channel. Z-stacked imaging (20 0.5 µm layers) showing 3-dimensions to the channel architecture. GFAP staining (TRITC) makes up a peripheral ring around the vascular central core of tissue, with some GFAP cells evident here. In the channel core, eGFP-MSCs (FITC) are again localizing to the vessel wall. The channel image is tilted forward on the Y-axis to demonstrate channel depth. Nuclei are counterstained with DAPI. Bar = 20 µm.

Channel core GFAP and S-100 double staining

Peripheral cells stained with GFAP indicating reactive astrocytosis along the inner surface of the polymer channel wall. GFAP positive cells were also noted within the channel core, showing the presence of atrocities in this compartment. Double immunostaining with GFAP (TRITC) and S-100 (FITC) was done to determine the viability and location of Schwann cells. In many GFAP positive cells in the channel core, GFAP staining co-localized with S-100 staining, demonstrating Schwann cell infiltrate. Other GFAP positive cells were present without having S-100 antigen.
Figure 3.17: GFAP and S-100 colocalization demonstrates the presence of Schwann cells within the core of the channel of a Schwann cell seeded scaffold. The central core is shown in closer detail (left) demonstrating clear colocalization of signal along with some cells that stain with GFAP alone. GFA and S-100 colocalized on the cell population occupying the channel core, but not in the periphery, demonstrating 2 distinct cell population. 
Bar = 20 µm
The pattern of GFAP-S-100 co-localization was evident to a similar extent in each of the animal groups, regardless of whether Schwann cells had been placed in the scaffold channels initially. Extensive S-100 staining was seen in the core of channels (green) from Matrigel control animals (*Figure 3.18*, right). Schwann cell infiltration into the Matrigel channels must have occurred after 4 weeks in vivo. S-100 positive cells may therefore have migrated into the scaffold from myelated nerve roots at the CNS/PNS interface.

In eGFP-MSC loaded scaffolds the colocalization is seen, with GFAP in red (TRITC) and the S-100 stain in far red (Cy-5) (*Figure 3.19*)

*Figure 3.19:* In eGFP-MSC channels, GFAP (TRITC) is seen in a peripheral concentric pattern and a more punctate pattern in the core (A) where the staining colocalizes with S-100 stain (B) (Cy-5, pseudo-coloured to magenta). Bar=100 µm
**Vimentin immunostaining**

The intermediate filament Vimentin is upregulated in reactive astrocytes in CNS injury, albeit to a lesser degree than GFAP (Bushong et al., 2003). It is a marker of immature astrocytes (Bramanti et al., 2010), as opposed to GFAP expression in mature positive cells. Vimentin is also a component of the cytoskeleton of MSCs. Antibodies to Vimentin were used in channel sections to identify any staining patterns that might further define compartmentalization and stage the process of astrocytosis. In Figure 3.19, shows the core area of a Matrigel (A) and Schwann cell channel (B) Vimentin positive cells were seen to be densely concentrated in the channel core for each animal group. The peripheral compartment of the channel had scattered cells that expressed Vimentin. Towards the outer edge of the channel, cell staining was seen to intensify, representing a higher proportion of the cells here. In C, an MSC channel is shown. Fluorescent cells are integral to the blood vessel walls, circumscribing the lumen. As eGFP positive cells would also fluoresce at this wavelength, this makes it difficult to discern if the perivascularar cells are eGFP-MSCs or vimentin positive, or both.
**Figure 3:19:** Fluorescence related to Vimentin staining localizes to the channel core in Matrigel (A) and Schwann cell channels, and to the outer edge of the channel periphery. In (C), fluorescent cells are integral to and circumscribe the vasculature. Bar = 50 µm

It is possible that Vimentin positive cells in the channel core are the same cell as that which was GFAP positive. The relative predominance of Vimentin stain in the core as opposed to the periphery, where GFAP is conversely predominant, suggests an earlier stage in astrocyte maturation for those cells in the core, and a more established state of cell maturation in the outer channel compartment.

**Neuroglycan-2 Immunostaining**

Production of chondroitin sulphate proteoglycan (CSPG) species by astrocytes represent an established state of gliosis by which boundary borders are established. To test the hypothesis that variable stages of astrocyte maturation in response to the injury may be present respectively in the core and peripheral channel compartments, Matrigel and Schwann cell channels were stained with antibodies to the CSPG Neuroglycan-2 (NG-2). In the Matrigel channel (Figure 3.20) NG2 staining was seen to be more dense and uniform around the channel
periphery. In the channel core, the staining had a loose reticular pattern interspersed with scattered immuno-positive cells. The distinction between core and peripheral compartments was more clear in the Schwann cell channel, where the reticular core is abruptly demarcated from the uniformly staining peripheral zone.
Figure 3.20: Immunostaining for the CSPG Neuroglycan-2 in a Matrigel (A) and Schwann cell channels (B) demonstrates a reticular core pattern and more uniform granular pattern in the periphery. The different areas of staining are sharply separated in the Schwann cell channel by a boundary structure.

Channel area proportions of GFAP, S-100 and vimentin positive tissue

The proportion of GFAP, S-100, and Vimentin positive cells in a given channel was calculated using ImageJ analysis software. Emission wavelengths were separated, thresholds were set to eliminate background, and the images were converted to black and white. The proportion of channel area occupied by the stain to total channel area was measured as a volume fraction. For the Matrigel group, 91 separate channel images with GFAP and S-100 staining were analyzed and 84 Vimentin sections were imaged. For the SC group, 70 GFAP/S-100 channels were analysed, and 95 with Vimentin stains. In the MSC group, there were 72 images for GFAP and S-100, and 107 images of Vimentin.
Figure 3.21: The proportion of channel area staining with GFAP, S-100 and Vimentin in each animal group is presented as a volume fraction percentage. Bars represent the total average in individual channels. Comparison of means was done with the Kruskal-Wallis ANOVA and a Dunn’s Multiple Comparison of means post test.

As seen in Figure 3.21, a significantly higher percentage of GFAP staining was measured within Matrigel filled channels (30.19 +/- 1.5 %) when compared to eGFP-MSC (19.15 +/- 2.5%) and SC filled channels (17.10 +/-1.28%), p<0.0001. The result suggests that Matrigel alone in a channel allowed for astrocyte infiltration from the surrounding cord more freely than a channel that was already occupied by Schwann or stem cells. No significant difference was seen between astrocyte infiltration in the latter two groups.
There were no statistically significant differences between the Matrigel (5.32 +/- 0.37%), Schwann cell (7.31 +/- 0.77%) and eGFP-MSC (10.07 +/- 1.03%) with regard to the proportion of S-100 positive staining. The result is surprising in that one would expect the Schwann cell loaded channels to have a higher proportion of S-100 cells present. The S-100 positive population may have equilibrated across the 3 experimental groups due to endogenous cell migration. For Vimentin staining, no statistically significant differences were observed across the three animal groups, with measured proportions of cells being 20.50 +/- 0.79% in Matrigel channels, 17.02 +/- 0.40 % in eGFP-MSC channels, and 19.29 +/- 0.47 % in SC channels. One may have expected the eGFP-MSCs group to have a higher proportion of channel area occupied given scaffold cell loading.

The proportion of GFAP, S-100 and Vimentin positive cells at each of the quarter level was also analysed to determine if scaffold length had any bearing on the ability of cells to migrate and become established within the channel. As Figure 3.22 shows, there were no significant differences between proportional cell area occupied at any given level of the scaffold, and the results are in keeping with the total averages throughout the scaffold length.
Figure 3.22: The proportional area of a channel occupied by GFAP, S-100 and Vimentin positive cells at ¼, ½, and ¾ levels through the scaffold.
Quantification of the inflammatory profile within scaffold channels

To assess the degree of inflammation occurring within scaffold channels, quarter length sections through the scaffold were stained with antibodies to inflammatory cell types. Antibodies to CD45R as the common leukocyte antigen, CD3 (T-Cells) and the ionized calcium binding adapter molecule-1 (Iba1) specific to macrophages and microglia, were used across each animal group. In each animal group, the scaffold can be seen to be a highly inflammatory environment, with an established chronic inflammatory pattern evident at 4 weeks post implantation.

Figure 3.23 shows representative channels from the Matrigel (A), Schwann cell (B) and eGFP-MSC (C) animal groups, stained with antibodies to CD45 and developed with the AEC chromogen. CD45+ cells are present in high numbers, diffusely throughout each channel. In Figure 3.24, the staining pattern of microglia in each of the Matrigel (A), Schwann cell (B) and MSC (C) is more localized to the channel core. The cell type is present at the channel periphery participating with the structure of the interface with the polymer. That macrophages would be part of multinuclear aggregates is in keeping their immune role. In CD3+ T-cell staining (Figure 3.25), the cell type again is more localized to the channel core, seen more defined to the core in the Schwann cell channel (B) than the Matrigel (A). CD3+ staining in MCS channels, visualized in the far red spectrum, appears more sparse.
Figure 3.23: Channels from Matrigel (A), Schwann cell (B) and eGFP-MSC (C) scaffolds, stained with antibody against the CD45 common leukocyte antigen as an indication of the overall inflammatory environment. Bar = 50 µm
**Figure 3.24:** Channels from Matrigel (A), Schwann cell (B) and eGFP-MSC (C) channels, stained with antibody against the Iba1 antigen for microglia/macrophage identification. Cellular density in the channel core is high, and the cells participate as aggregates at the tissue-polymer interface. Bar = 50 µm
Figure 3.25: Channels from Matrigel (A), Schwann cell (B) and eGFP-MSC (C) channels, stained with antibody against the CD3 antigen for T-Cell identification. There is a higher core concentration of cells particularly in the (B). In (A) and (C), the core concentration is more diffuse, and cells can be seen to line the core-periphery boundary.
Each stained channel was independently imaged and analysed with NIH ImageJ software to measure the proportion of channel that was occupied by inflammatory cells. This was calculated as the percentage of channel area stained in relation to the total channel area, when the staining intensity was within the threshold set for the image group. For CD45, 93 separate images of stained channels were analysed over 3 scaffold levels in the Matrigel group, 117 in the eGFP-MSC group, and 107 in the SC group. For Iba1, 97 Matrigel channels, 121 MSC and 110 SC channel images were analysed. For CD3 the respective numbers were 95 Matrigel, 115 MSC, and 102 SC channels.

Figure 3.26 details a comparison of the total average proportion of inflammatory infiltrate as a fraction of the channel area and as the average of all measurements across all three levels. For CD45, highly significant differences (p<0.001) existed between animal groups, with the Schwann cell scaffold demonstrating less leukocyte infiltrate (15.98 +/- 0.57 % of total channel area) when compared to Matrigel only (24.31 +/- 0.94 %) and the eGFP-MSC group (32.60 +/- 0.776 %). It is also notable that for the MSC group, nearly 1/3 of the channel area was made up of inflammatory cells.

The proportion of microglia was analysed using antibodies against Iba1. Highly significant differences (p < 0.0001) were seen in Iba1 staining comparisons between animal groups. The Schwann cell group overall had more microglial cell infiltrate, comprising of 43.78 +/- 0.85 % of total channel area, compared with 27.39 +/- 1.32 % if the Matrigel channel area, and 34.11 +/- 0.80 % of the eGFP-MSC filled channel. In regards to CD3 staining, again the Schwann cell group demonstrated less T-Cell infiltrate than the Matrigel group with a proportional area of 8.35 +/- 0.64 % compared to 15.98 +/- 0.83 % (p<0.0001).
**Figure 3.26:** The inflammatory profile of scaffold channels. The proportion of the area within a channel that is occupied by inflammatory cells is compared across animal groups. Image analysis of the area stained as a proportion of the total channel area yields the percentage of area occupied. (*** p<0.0001)

CD3 positive cells were seen to be significantly reduced in number in the MSC group (4.10 +/- 0.484 % of total channel area) compared to the Matrigel group (15.98 +/- 0.832 %, p<0.0001). There was a trend towards less CD3+ cells when comparing MSC and SC channels, but there was not a statistical significance. SC channels did themselves have significantly less CD3+ cell infiltrate (8.38 +/- 0.649 %) than did the Matrigel group (p< 0.05).
Inflammatory Profile of Scaffold Channels

**Figure 3.2:** The inflammatory profile of CD45, Iba1 and CD3 cells by scaffold level.
The inflammatory profile was also analysed by scaffold level within a particular animal group, to determine if there were any differences in cell access to various scaffold regions, or in the ability of the infiltrate to migrate through the scaffold length. When compared with the Kruskal-Wallis ANOVA and a Dunn’s Multiple Comparison of means post test, no significant differences between the proportion of infiltrate measured at a particular level within an animal group were observed for any of the three markers used. The proportion of CD45 cells measured at the halfway point of the Matrigel scaffold were not significantly different to each of the levels observed within the eGFP-MSC animals, but were significantly more than the each of the levels within the SC animals (p<0.05). Consistent with the averaged proportions for the entire scaffold, significant differences were seen between each level of the SC group and all other levels in the other animals. For Iba1, all levels of the Schwann cell group had highly significant (p<0.0001) increases over each of the levels seen within the Matrigel group. Only the midway point of the Schwann cell scaffold had measurements that were consistently and significantly increased over those seen at each of the eGFP-MSC levels (p<0.05).

3.3.4 Quantification of Axonal Regeneration through the Scaffolds

The numbers of axons regenerating through the scaffold implants in each group (n=6 per group) were identified by neurofilament staining in 8 micron transverse sections and counted by a blinded observer. Counts were made at lengths of one quarter, one half and three quarters the distance through the scaffold from the rostral to the caudal cord interfaces, as represented in the schematic figure (above left).
**Figure 3.28:** Neurofilament staining at quarter lengths through the scaffold. Left is a 4x low power overview of an eGFP-MSC section showing the preservation of the 6+1 channel architecture during sectioning. The hydrogel is dissolved during tissue processing. Around the periphery of the scaffold the fine capsule can be seen, along with 2 areas of nerve fascicle staining (at 11 and 3 on the clock). These areas act as useful internal controls for staining efficiency. In (A) there is a representative channel from a control scaffold, with few scattered axons confined to channel core compartment. In (B), a channel from a Schwann cell scaffold shows a more dense arrangement of neurofilament staining, again confined to the channel core. In (C), an MSC channel demonstrates significantly less neurofilament staining with an increased uptake of non-specific staining, typically seen in macrophages with the DAB chromogen.

**Axon counting and frequency distribution**
Axons were stained with monoclonal primary antibody to neurofilament protein, with a biotinylated secondary antibody and use of the DAB chromogen for
Axonal Regeneration Supported by OPF+ Cell-Loaded Hydrogel Scaffolds

visualization. Some sections were lightly stained with Haematoxylin. The axons appear as a pinpoint, dark brown pattern within the channel core (Figure 3.28 A-C), and are counted under 40x power primarily, or 100x to distinguish individual fibres within axonal bundles or to distinguish axons from macrophages.

**Figure 3.29:** Histogram analysis to determine the frequency distribution of axonal counts in ‘bins’ of 200 axons, in control (upper panel) and in Schwann cell animals (lower panel)

To determine if the distribution of counts was Gaussian or non-Gaussian, a histogram analysis was done with ‘bin’ number groups of 200 axons for the control and Schwann cell groups (Figure 3.29). The analysis is done across the 6 animals and at each of the three levels of the scaffold, for a total of 18 count of observations. The upper panel for control animals shows the frequency distribution of count numbers to be non-Gaussian with a skew bias towards the left and a tail to the right.

In the lower panel, there is a bimodal, non-Gaussian distribution axon counts in the Schwann cell group. Two animals receiving SCs showed a
regenerative capacity similar to the control animals, while four animals demonstrated improved growth over the control (again with a skew to the left). This analysis is important in that direct comparison of axon numbers must be a comparison of median values (non-Gaussian distribution) and not a comparison of means (Gaussian distribution).

**Comparison of Matrigel, Schwann cell and eGFP-MSC scaffolds**

Quantification of axon numbers comparing control to Schwann cell groups, shows significantly more axons regenerating in the Schwann cell group (p<0.01). The median number of regenerating axons counted at each scaffold level (1/4, ½, and ¾ counts combined as independent observations) in the control group was 270 axons compared to the median axon number of 1313 in the Schwann cell group (n=18). The median axon count in the eGFP-MSC group was 12 axons, significantly less than both control and Schwann cell with a p value <0.001.

**Total Axon Counts at Each Scaffold Level**

*Figure 3.30* A comparison median axon counts per level in 6 animals per group, and at 3 levels per animal, representing the total number counted throughout the scaffold
When the observations of axon numbers are limited to one level through the scaffold, there are no significant differences between axon counts within the same animal group. The results suggested a uniform penetration of axons through the length of the scaffold despite whether the counts were high or low for the animal. Within the control group, median axon counts were 432.2, 282.8 and 265.8 at the \(\frac{1}{4}\), \(\frac{1}{2}\), and \(\frac{3}{4}\) levels respectively (n=6 per level). Within the MSC group, median axon counts were 47.83, 36.50, and 29.00 at the \(\frac{1}{4}\), \(\frac{1}{2}\), and \(\frac{3}{4}\) levels respectively (n=6 per level). For the Schwann cell group the media values were 1253, 1220, and 1150 respectively. There were significantly more axons at each level (n=6) in the Schwann cell group compared with the control group (p<0.001), suggesting again that the Schwann cell scaffold is more permissive to or supportive of axonal regeneration over a Matrigel loaded scaffold. However, the significant differences between control and eGFP-MSC scaffolds were lost when a direct comparison of axons at each level was made independently (p>0.05).

**Figure 3.31** A comparison of median axon counts in 6 animals at a given quarter level through the scaffold.
3.3.5 **Quantification of Scaffold Blood Vessel Supply**

An unbiased, stereologic approach was taken to estimate the volume fraction, length density and surface density of vessel structures within the scaffold channels of each animal group. Sections of scaffold tissue taken at ¼ lengths through the scaffold of each animal were stained with antibodies to Collagen IV to identify the vessel walls and distinguish them from cystic structures. A green FITC secondary was used with Matrigel and SC sections, and a Far Red secondary was used on MSC channels given the conflict between a FITC secondary and eGFP from the MSCs. Images of each channel were then captured and analyzed using stereology counting grids.

Figure 3.32 demonstrates representative vessel staining in Matrigel (A), Schwann cell (B) and eGFP-MSC (C) channels. In all channels, vessels are largely confined to the channel core compartment. Vessels in Matrigel are of relatively low density, and of only moderate size. A more dense vessel network is seen the Schwann cell channel core, consisting of numerous small diameter vessels with high central concentration. In MSC channels there are less vessels in number, but these are of relatively large calibre, where the smallest observed would be on par with the largest seen in the Schwann cell or Matrigel channels.
Figure 3.32: Comparison of the vascular beds of Matrigel (A), Schwann cell (B) and eGFP-MSC (C) channels. The notable findings are a relative avascularity in the Matrigel channels compared to a high density of small capillary-like vessels with thin wall structure in the centre of the Schwann cell channel. MSC channels are as previously noted, large, thick walled vessels of wide diameter dominating the channel architecture. Bar = 50 µm.

Stereology grids were copied onto a transparency and laid over each channel image for analysis. For volume fraction, a simple point grid was used to determine the proportion of unit volume in the channel reference space. For length density, a grid of unbiased counting squares of known area was used to count the number of vessel profiles in the reference space. For surface density, a series of linear test probes was used to count the number of intersections of vessel structures with lines of known length within the reference space. Between 25 and 30 channels were available for analysis across each animal group, yielding very large numbers of vessel and reference point observations.
**Volume fraction (Vv)**

Volume fraction (Vv) represents an estimate of the volume of vessel features as a proportion of the channel volume. Each available channel was analyzed at the quarter length interval for each of the 6 animals per scaffold group. For Matrigel there were 33, 33, and 27 channels counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths respectively across the 6 animals, providing a total number of 377 feature and 10,853 reference points for the group. For SC scaffolds 29, 35 and 22 channels were counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths, providing 1638 feature and 8307 reference points. For MSC animals, 28, 35, and 28 channels were counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths, providing 1547 feature and 9323 reference points.

As seen in Figure 3.33 (A), the mean volume fraction for vessels embedded within Matrigel channel sections was 0.034 +/- 0.004 per channel as the summation of all channels, without significant changes noted along the quarter length intervals. For SC channels, the mean vessel volume fraction per channel was 0.197 +/- 0.010, without significant variability across quarter lengths, and for MSC channels the vessel volume fraction was 0.166 +/- 0.014. Using 2 way ANOVA analysis, statistically significant increases in volume fraction were observed with SC and MSC channels over Matrigel control (p<0.0001), while vessel volume fraction was comparable between MSC and SC channel groups.

**Length density (Lv)**

Length density (Lv) is a measure of the total length of vessel objects within the reference channel space and has important implications for the diffusion capacity of the vessel compartment. For Matrigel there were 34, 33, and 25 channels counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths respectively across the 6 animals, providing a total number of 598 vessel profile and 709 reference points for the group. For SC scaffolds 32, 34 and 25 channels were counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths, providing vessel feature and 702 reference points. For MSC animals, 35, 29, and 30 channels were counted at \( \frac{1}{4}, \frac{1}{2}, \) and \( \frac{3}{4} \) lengths, providing 848 feature and 680 reference points.

In Figure 3.33 (B), length density for vessels in Matrigel channels was \( 0.469 \times 10^{-3} +/- 0.039 \times 10^{-3} \ \mu m^{-2} \) per channel, and in MSC channels was \( 0.693 \times 10^{-3} +/- 0.049 \times 10^{-3} \ \mu m^{-2} \). No significant difference was observed between the vessel length densities in Matrigel and MSC channels. A significantly higher
proportion of total blood vessel length was present in SC channels, with a mean \( L_v \) of \( 1.655 \times 10^{-3} \pm 0.114 \times 10^{-3} \ \mu m^{-2} \) per channel, indicating that a higher in the SC channel sections. No significant differences were observed across the quarter length intervals of scaffolds suggesting uniform penetration and distribution of vessel segments down the scaffold length.

**Surface density (Sv)**

Vessel surface density (Sv) was calculated as the proportion of the channel surface area that was made up by a vessel structure. For Matrigel there were 33, 35, and 25 channels counted at \( \frac{1}{4} \), \( \frac{1}{2} \), and \( \frac{3}{4} \) lengths respectively across the 6 animals, providing a total number of 903 vessel-line intersections within 3690 reference space points for the group. For SC scaffolds 32, 35 and 27 channels were counted at \( \frac{1}{4} \), \( \frac{1}{2} \), and \( \frac{3}{4} \) lengths, providing 2735 vessel-line intersections and 3793 reference points. For MSC animals, 29, 35, and 30 channels were counted at \( \frac{1}{4} \), \( \frac{1}{2} \), and \( \frac{3}{4} \) lengths, providing 1573 feature and 3307 reference points.

Figure 3.33 (C) details the analysis, whereby the vessel surface density was \( 0.816 \times 10^{-2} \pm 0.054 \times 10^{-2} \ \mu m^{-1} \) per Matrigel control channel in both SC and MSC channels, \( 2.402 \times 10^{-2} \pm 0.132 \times 10^{-2} \ \mu m^{-1} \) and \( 1.585 \times 10^{-2} \pm 0.159 \times 10^{-2} \ \mu m^{-1} \) respectively. Sv of SC at each quarter and all scaffold intervals combined was significantly increased over both Matrigel control (p<0.0001) and MSC channels when all MSC intervals were combined (p<0.0001). In the quarter interval analysis, the relationship between MSC channels and the other 2 groups was complicated by an increase in the Sv at the MSC \( \frac{3}{4} \) interval. At that interval only, the difference between MSC and SC was not significant, with a significant decrease in MSC Sv over SC channels noted that the \( \frac{1}{4} \) (p<0.01), \( \frac{1}{2} \) (p<0.001). Sv of MSC channels was statistically unchanged over control at the \( \frac{1}{4} \) and \( \frac{1}{2} \) interval, but significantly increased at the MSC \( \frac{3}{4} \) length (p<0.001), sufficient to bring the SV of all MSC channels to significance over control when combined.
**Figure 3.33:** Volume fraction ($V_v$), Length Density ($L_v$) and Surface Density ($S_v$) for scaffold channel sections at quarter length and in total (A-C) are shown with the corresponding calculations for physiologic values (D-F).
Applications of Vv, Sv and Lv estimates to the scaffold model

Given that the mean reference volume is known for each animal and at each scaffold section, fractional volumes are readily converted to physiologic values that are relevant to channel sections. The mean channel volume for 8 µm section varied from animal to animal and different scaffold lengths, but it was possible to correlate vessel data obtained from the corresponding animal and scaffold length. From the vessel volume fraction, the mean vessel volume per channel section was calculated (Figure 3.29 (D)). This value could approximate the volume of blood within a channel section.

In Matrigel channels, the mean blood vessel volume was 28,770 +/- 2894 µm³. This volume was significantly less than both the Schwann cell channel vessel volume (131,688 +/- 15,399 µm³, p<0.01) and the eGFP-MSC vessel volume (161,349 +/- 11,898 µm³, p<0.001). No significance difference was seen between the Schwann cell and MSC channel. In Figure 3.29 (E), the mean vessel length per scaffold channel section is shown. Schwann cell channels had a significantly longer expanse of vessels (1175 +/- 147 µm of length per 8 mm channel section, p<0.001), than Matrigel (412 +/- 41 mm) and MSC channels (564 +/- 44 mm) despite the equivalent vessel volume of MSCs. In Figure 3.29 (F), the mean vessel surface area per channel is shown for each animal group. Schwann cell and MSC had statistically equivalent vessel surfaces on the channel face, being 20,404 +/- 2231 µm² and 13,508 +/- 819 µm², with both having higher surface areas than Matrigel (7,875 +/- 662 µm², p<0.001 and p<0.01).

Put together, the results indicate that whereas MSC channels maintained their blood volume by means of larger vessel calibre, Schwann cell channels had an equivalent surface area and vessel volume by virtue of high numbers of smaller vessels contributing to the high length density.

Mean vessel diameter and mean cross-sectional area of vessels.

Other important physiologic values are derived from the fractional estimates. Mean vessel diameter is an estimate based upon the ration of surface to length density. This parameter has direct implications for the blood flow rate through a vessel network, given the flow is inversely proportional to the radius of the vessel by its fourth power. In Figure 3.34 A, mean vessel diameter was
statistically larger in MSC channels, corresponding to the visual observations. The mean diameter of MSC vessels was on the order of 8.11 +/- 0.67 µm, significantly wider than Schwann cell vessels (4.67 +/- 0.44 µm, p<0.001). That the Schwann cell mean vessel diameter is half as small as the MSC vessel mean would increase the rate of blood flow in a Schwann cell channel by 16 times. The diameter of Matrigel vessels was statistically equivalent to both Schwann cell and MSC channels, being intermediate to the two at a mean diameter of 6.44 +/- 0.52 µm.

\[
\bar{d} = \frac{S_v}{L_v \pi}
\]

\[
\bar{a} = \frac{V_v \pi d^2}{L_v} \cdot \frac{1}{4}
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**Figure 3.34:** Mean vessel diameter (A) and vessel cross sectional area (B) is calculated for channel sections, having important implications for blood flow.
Mean vessel cross-sectional area is calculated as a ratio of vessel diameter and fractional volume to length density (Figure 3.34 B). MSC mean vessel cross sectional area far exceeds the other two groups, measuring $20,283 \pm 5141 \ \mu m^2$ ($p<0.001$), nearly a factor of 10 times that of the Schwann cell and Matrigel groups. The latter groups have mean areas ($2392 \pm 545 \ \mu m^2$ and $3120 \pm 883 \ \mu m^2$).

**Vessel radial diffusion diameter.**

The radial diffusion coefficient is a robust, stereologic measure of a cylindrical zone of diffusion around a blood vessel. It is calculated as the inverse function of the length density. The calculated values for Matrigel, Schwann cell and MSC channels are shown in Figure 3.35. The radial distance of diffusion around Schwann cell vessels was significantly smaller than the other two groups given the high density of unbiased vessel counts in these channels. The diffusion distance for Schwann cell vessels was $198.7 \pm 16.41 \ mm^2$ ($p<0.001$) compared to $884.0 \pm 97.6$ in Matrigel channels and $498.8 \pm 35.5$.

![Vessel Radial Diffusion Distance](image)

$r_{(diff)} = \frac{1}{\sqrt{\pi \cdot Lv}}$

**Figure 3.35:** Mean radial diffusion distances in the vessel beds of Matrigel, Schwann cell and MSC scaffold channels.
Axon number and vessel fraction correlations

To determine if there was any statistically significant correlation between the vessel parameters and axon numbers, means of axon counts and vessel calculations were tabulated from corresponding scaffold intervals in corresponding animals. Given that Matrigel axon counts were low, and axon numbers in Matrigel substrate were augmented by the addition of Schwann cells, all animals in these two groups were included in the analysis to provide sufficient variability. Significant linear correlations (Figure 3.36) were seen between the axon counts at a given scaffold level and the corresponding vessel length density (Spearman $r = 0.7852$, $p<0.0001$), vessel surface density (Spearman $r = 0.7719$, $p<0.0001$), vessel volume fraction (Spearman $r = 0.6862$, $p<0.0001$) and the mean vessel diameter (Spearman $r = -0.4635$, $p = 0.0113$).
**Figure 3.36:** Axon counts are seen to significantly correlate to vessel length density, surface density, volume fraction and mean vessel diameter in a linear manner at a given scaffold level. All Matrigel and Schwann cell animals were included in the analysis (n=29 observations across 18 animals).

Given that the tightest correlation was seen between axon number and vessel length density, and given the significance of decreasing vessel diameters at higher axon numbers, the relationship between axon number and vessel radial diffusion distance was calculated. The correlation was also of statistically significant, with a Spearmann r of -0.7852 p<0.0001. The relationship function was observed to be hyperbolic. The relationship will likely be of tremendous significance as we attempt to improve axonal densities in the tissue-engineered spinal cord.

**Figure 3.37:** The correlation between axon number and vessel radial diffusion distance implies a hyperbolic relationship, whereby increasing the axonal numbers relates to decreasing diffusion distances that approach but cannot be zero.
3.4 Discussion

3.4.1 The Influence of Scaffold Structure on Experimental Analysis in a Spinal Cord Transection Model

In the present study we have shown significant advancement in the precision with which the physical or structural variables of the biomaterial can be controlled. We have utilized a hydrogel of demonstrated biocompatibility in the cord, whose surface area has been modified with positive charge, and whose structure was seen to be highly reproducible. In addition to its good surgical alignment in vivo, scaffold integrity as a micro-environment for regeneration remained intact for the duration of the experiment. The scaffolds became encapsulated, and we were able to comment on the microscopic structure of this fibrous capsule, as well as the presence of normal anatomical structures surrounding the implant such as the spinal cord nerve roots. The spatial orientation of channels relative to each other was accurately maintained according to the scaffold design. Where disruptions in the scaffold architecture were seen, these typically occurred in a predictable manner relating to the process of cutting sections through the scaffold material, or by the growth of tissue in from the rostral and caudal ends of the cord. A major advancement was that individual channels could be treated as separate observations, allowing for high numbers in the analysis. Channels remained intact throughout their length. Only rarely did we see tissue growth between channels, usually when there were small tears in the hydrogel material or again at the scaffold ends. The average number of intact channels per scaffold available for analysis was 5 out of the 7, and in up to 40% of scaffolds we could observe all 7 channels at each quarter level of the analysis. The average number of observations available did not vary at the ¼, ½, or ¾ levels.

Scaffold channel area within any animal group had less than 2% variability. We could readily measure the effects of tissue fixation on channel area. Channel area across the 3 animal groups was also consistent, as there was no significant difference between SC and Matrigel scaffolds; however, the model was sensitive enough to demonstrate differences between the channel areas of scaffold loaded with MSCs and the other two groups. We postulate that this might reflect
the relative effects of tissue fixation shrinkage, given that the MSC channel were filled with a much looser, more open type of tissue. We have clearly shown that individual channel sections can be treated as microenvironments which can then be averaged to discern trends in the behaviour of the regenerating tissue. Multiple examples are shown here, in structural analysis, volume fraction of cell infiltration, axon counting, and detailed analysis of vessel structure. Discrete channel lengths across animals can be analyzed in parallel. Correlation analysis has been shown to be possible and highly informative, when different types of analysis (axonal versus vascular) are done in corresponding animal lengths. Meticulous record keeping and a dedicated system of channel imaging as made this possible.

The types of analysis which can be done will be further expanded. Given that a particular channel can be consistently identified by landmarks on the section, the channels allow for observations down individual length. In the future will be possible to quantitate linear growth density of axons, for example, or to track cell infiltrate into a channel over time. Differential axonal density at varying lengths may provide clues to the directionality of growth. Scaffolds were not cut longitudinally in this study, but we have plans for a series of animals to assess cell or axon migration into the channels from the surrounding cord over a course of time. It will be possible to track axons through individual channels in conjunction with Fast Blue axon tracing as the lab has previously shown (Chen et al., 2009). Finally, as we further explore neurotrophic cell lines, the ability to track individual scaffold channels along their length may give insight into predominance of dorsal (sensory) or ventral (motor) axonal growth in response to a particular growth factor. This may enable scaffolds to provide regionalized support of axonal growth by placing specific cell lines into specific channels.

3.4.2 Scaffold Channels have Structural Compartments

This is the first study that we have done which analyses the cellular architecture within regenerating scaffold channels. Scaffold channels were seen to be highly cellular, viable environments, in which there no evidence of tissue necrosis. Each animal group demonstrated similarities in the architecture of the channel, yet had particular characteristics of tissue structure which varied
depending upon the cell type loaded into the scaffold. Matrigel channels could be considered as a useful control given that any cellular component must be derived from the surrounding cord environment. Matrigel itself essentially then serves as the migratory substrate, the extracellular matrix along which cells move into the scaffold, become resident and divide. The Matrigel channel in that sense probably represents an “empty canvas” so to speak, or how the rat transected spinal cord will react to heal in the absence of any cellular or molecular cues from within the scaffold, and with plenty of space into which a new cellular environment could be established. It is a baseline environment onto which the details can be added by introducing other variables, such as other cell types, which of course are themselves suspended in Matrigel. We have not experimented with different cell suspension matrices such as fibrin; to date we have kept this as a constant across animal groups and various regeneration projects. Matrigel itself is capable of supporting a regenerative environment to a minimal degree. Once the optimal cell has been settled upon, it may be of interest to examine the influence channel or cell suspension substrate might have on channel tissue formation.

The results shown provide a progression of tissue structure analysis, from a generalized histopathology overview using H&E, Masson’s Trichrome, and eosin autofluorescence to detailed identification of cell types in the channels using immunohistochemistry. At 4 weeks, Matrigel filled channels demonstrated a central circular core of loose tissue that was circumscribed by a dense layering of tissue that extended to the periphery of the channel. Within the core were open small cystic areas and endothelialized vessels. At the periphery there were very few vascular structures or spaces, and there was evidence of foreign body type reactions such as multi-nucleated giant cells at the interface with the polymer. Using eosin autofluorescence to detail the fibrous microarchitecture of elastic fibers and cellular basal lamina, the channel was more clearly seen to have spatial compartments separate into central core and peripheral. These compartments were themselves physically separated by an intermediate layer, an internal banding of highly florescent fibrous structure.

In scaffold channels loaded with Schwann cells, the two separated compartments were again apparent. The cellularity of the core was more homogeneous, and of greater density than in the Matrigel channels. The channel is already full of cells at a time surgery, leaving relatively less room for cell
migration. Constituent cells that are not Schwann cells again must have been derived from the surrounding cord environment, and there must have been some transition of cell population from virtually all Schwann cell to the cellular composition seen at four weeks. The vasculature in Schwann cell channels was similarly confined to the core. Eosin autofluorescence demonstrated a characteristic ultrastructure in the core not seen in the other groups, whereby small open circular spaces were supported by stellate structures. A differential was postulated to include tissue supportive of small blood vessel beds, nuclei surrounded by stellate cytoskeleton, reactive astrocyte cytoskeleton, axonal tracts surrounded by supportive cells, such as Schwann cells. In retrospect, and in light of the vascular structure observed in the Schwann cell channels in the vessel stereology study, I would favour the eosin fluorescent pattern as relating to the vessel bed. Further work to differentiate or firmly identify the structures, such as to combine NF, GFAP, DAPI, or Collagen IV immunofluorescence with eosin is complicated by the fact that eosin fluoresces in both the FITC and TRITC wavelengths. However, a combination of AEC or DAB chromogen and eosin fluorescence would be straightforward.

In channels loaded with MSCs, separate core and peripheral compartments were again seen, but there was an entirely different architecture to the core. Overall the density of cells was less, the tissue was loose and open, and most strikingly there was an abundance of vessels of various caliber, sometimes with diameters of over 10 microns. Of the three animal groups, tissue formed within MSC loaded channels had the lowest elastic tissue content, based on the calculated surface area of eosin fluorescence. On review by Dr Joseph Parisi, a Consultant Neuropathologist at Mayo Clinic, the tissue had appearance similar to granulation tissue, such that would be formed outside the central nervous system, but not seen in the CNS. On the basis of the pathologic appearance, we initially put forward a hypothesis that MSCs contribute to the formation of tissue granulation tissue in the CNS. This tissue more typically ‘mesenchymal,’ found predominantly in dermal wound healing, as not in association with neuroectodermal tissue seen in the normal CNS or CNS pathologic states.
3.4.3 Scaffold Channels and the Peripheral Glial Compartment

Having noted structurally separate compartments within scaffold channels, we set about using immunohistochemistry analysis to identify what cell types may have contributed to each compartment. The peripheral laminar layer was shown in each animal group to stain with antibodies against the astrocyte marker GFAP using both AEC and fluorescent secondary antibody substrates. A significantly higher proportion of channel area was occupied by GFAP positive cells in the Matrigel group (30%), than that seen in MSC and SC groups (20%). The result again likely reflects the availability of space within Matrigel channel for a process of migration and cell division of innate CNS cells compared to a cell-loaded channel.

Glial cordones

Astrocytes are thought to play a role in developing compartmental boundaries in the CNS. Such boundaries abound in the CNS, segments, midlines, tract nuclei, functional columns, discrete axonal arcs or pathways, and the like. Increased GFAP staining has been observed in developmental boundaries such as the thalamic nuclei, and barrel cortical structures (Bushong et al., 2003). The extracellular matrix demarcating these boundaries (particularly rich in J1/tenascin glycoprotein) is believed to be laid down by radial astrocytes. Such structures were termed ‘glial cordones’, and may be dynamic entities of specialized glia secreting glycoconjugates (a mixture of glycoproteins, glycolipids, and glycosaminoglycans) which disappear after stable synaptic formation (Steindler, 1993).

If we are to liken a regenerative environment to a return to a developmental state, then such structures are seen to be important to redevelopment following injury. For example, spatial and temporal distributions of tenascin during development suggest a role in the guidance of neurons within functional patterns (Laywell et al., 1992). Following trauma to the cortex, discrete populations of reactive astrocytes upregulate the transcription and expression of tenascin (Brodkey et al., 1995). A recognized hallmark of reactive astrocytosis is the upregulation of intermediate filaments, primarily GFAP to a lesser degree vimentin, and the re-expression of nestin. This network of intermediate filaments
becomes very prominent in the cell body and in the cellular processes that hypertrophy and extend to form gap junctions with adjacent astrocytes (Bushong et al., 2004). Such networks of astrocytes are thought to distribute nutrient and ionic/buffering support to the area of injury, as well as limiting the effects of the free radical oxygen formed in areas of hypoxia via glutathiaone dependent mechanisms. There is evidence that GFAP and vimentin upregulation and filament assembly may influence axonal growth as well. (Menet et al., 2000, 2001) reported in vitro that astrocytes derived from transgenic mice homozygous deficient in both vimentin and GFAP, or in GFAP alone, were a better substrate for the outgrowth of neurites than wild-type astrocytes. The same group has shown in a lower thoracic spinal cord hemisection model (Menet et al., 2003) that axonal sprouting was increased and functional recovery was improved in GFAP--/Vim-/- mice compared with wild-type controls.

Within the channels of our scaffolds, we see no axonal growth present or extending into the peripheral compartment. Through a combination of repulsive and permissive interactions, radial astrocytes may influence the distribution of neurons and their processes within compartment interfaces (Faissner and Steindler, 1995). Neuronal guidance molecules such as the integrin, cadherin, and ephrin receptor families may be important (Redies, 2000; Tarone et al., 2000), as well as isoforms of the neural cell adhesion molecule (N-CAM). N-CAM in particular is upregulated during development and trauma and is involved in axonal guidance, synaptogenesis and its plasticity during regeneration (Zhang et al.,; Klementiev et al., 2008). In a detailed analysis of the laminar boundary zones of the dentate gyrus, (Bushong et al., 2003) have shown that astrocyte distribution conforms to a differential expression of N-CAM and the ephrin EphA4 between layer interfaces particularly in the inner and middle layers. The distribution was influenced by a functional boundary separating afferents of the commissural and perforant pathways.

**Polymer surface charge and astrocytosis.**

It is in the peripheries of the channel, along the surface of its inner circumference, where the effects of positive charge integrated into the substance of the OPF polymer (as amine residues) would presumably be most in influence. The principle of incorporating positive charge into the polymer was based on the
observations of improved axonal outgrowth and myelination in vitro on the charged OPF surface (Dadsetan et al., 2009). It was hoped that the positive charge may attract sprouting axons out to the peripheries and allow for greater numbers of regenerating axons to fill the channel. In vivo it may rather be the case that the first cell types to migrate or to extend into the injured area would have access to the positively charged surface and selectively attach to it. As reactive astrocytosis is a primary cellular event in the injury response, it would make sense that these cells arrive first, preferentially bind the charged surface and subsequently line the channel wall. To explore this possibility, we have several animals already processed in a time course design of weekly intervals post implantation, whose scaffolds will be cut longitudinally to assess migration events.

If the outer periphery becomes resident with astrocytes first, as a primary injury response event and as influenced by the positive charge of the polymer, we hypothesized that we might observe that area of astrocytosis to be at a more advanced stage in the process of gliosis. To that end we used two additional markers, Vimentin and Neuroglycan-2 (NG-2). During early development, radial glial and immature astrocytes express mainly Vimentin, which is seen to be progressively replaced by GFAP in differentiated astroglial cells (Bramanti et al., 2010). NG-2 is upregulated by 24 hours post injury in the spinal cord, and reach peak levels in quantitative western blot analysis in stab injuries within 8 days post injury (Tang et al., 2003; Galtrey and Fawcett, 2007). We noted that the distribution of vimentin staining in each of the animal groups localized more to the channel core suggesting that immature astrocyte phenotypes predominated there. Vimentin positive cells were present peripheral layers, as we would expect in upregulation associated with injury. However the primary stain present in abundance in the periphery was GFAP, suggesting a more mature astrocyte phenotype. Neuroglycan-2 was present throughout the scaffold channels of Matrigel and Schwann cell groups, in a centralized loose reticular pattern separated from the peripheral pattern, which was more dense, homogeneous and granular.
The scaffold channel periphery and axon growth

The peripheral layer of more mature astrocytes may direct the organization of newly regenerated axons to occupy or infiltrate into the central part of the channels either by providing contact guidance for the axonal growth or perhaps by exclusion or growth inhibition from the peripheral zone by established proteoglycan barriers. These influences may allow higher numbers of nerve fibers to extend across the entire channels of the scaffold in a more organized fashion. When we compared scaffolds of varying polymer types (Chen et al.), axonal growth through charged OPF was seen in centralized pattern that showed a significantly higher number of regenerated axons compared to a dispersed pattern of growth seen in uncharged polymers. In each polymer type, a circumferential rim of tissue was seen surrounding the core. In the dispersed pattern, lower densities of axons were spread more widely across the channel diameter. At times in uncharged groups, axons were seen to grow tangentially to the longitudinal plane of the channel. Another recent study using an in vivo hemisection model of spinal cord injury assessed the ingrowth of neuronal tissue elements in hydrogels with different charges. Neurofilaments were similarly demonstrated to infiltrate the central part of the hydrogels most plentifully in the copolymers with positive charges (Lesny et al., 2006). The structure of the scaffold in terms of its channel numbers may also influence the dispersion of axonal growth. (Yao et al.).

Based on these findings, we now propose the hypothesis that we may have created ‘glial cordone’ equivalents within the channel microenvironment. Surface charge of the scaffold wall may be more important for the organization of a laminar boundary of more established astrocytes, which in turn is influencing boundary formation and the distribution and growth of axons. Our model therefore may allow for further investigations into the dynamics of reactive astrocytosis and its influence on neuronal growth and guidance. We have shown the feasibility of detailed immunohistochemical analysis of preserved regeneration tissue columns. Tenascin would an intriguing start, as would matrix proteins of importance such as N-CAM, and ephrin. Modification of cell adhesion molecules in the injured cord is emerging as a promising approach for regeneration gene therapy (Lavdas et al.).
3.4.4 Scaffold Channels and the Central Regenerative Core.

If scaffold charge and the reactive astrocyte periphery can organize or direct axonal growth to a central core, the core remains as the area in which implanted cells would presumably most influence the architecture for regeneration. We observed that there must have been transition of cell types in the scaffold, from a high proportion upon scaffold implantation to a relatively low proportion limited to the central core at the experimental endpoint. Implanted cells might have a short time to act to influence the regenerative environment but differences in the cellular architecture between the groups were seen, as discussed above. The capacity for tissue formed in response to cell seeding to support axonal extension through the scaffold was incremental across the animal groups. Here we discuss possible relationships between the cell type implanted and its influence on regeneration.

Axonal regeneration in the core

Axons were only seen in the core compartment in scaffold channels. We have proposed they are excluded from the periphery by established gliosis. Axonal counts were made by visualizing stained neurofilament. The counts were seen to have a non-Gaussian distribution and accordingly are reported as median numbers. Median total axonal counts in this study for Matrigel (270 axons per scaffold) were equivalent to that seen in PLGA scaffolds with Matrigel (Olson et al., 2009). Median axonal counts for Schwann cell loaded scaffolds (1313 axons per scaffold) were also comparable to that seen in OPF+ channels in parallel studies (Chen et al., ; Rooney et al., 2011). Preloading the scaffolds with MSCs resulted in virtually no axonal extension into the scaffold, significantly less than even the Matrigel control. This result is also consistent with our previous observation in OPF+ scaffolds containing cAMP-eluting microspheres (Rooney et al.). Whether the channel tissue formed in the presence of MSCs actively inhibits axonal growth or simply is not a substrate capable of supporting axon extension has not yet been clarified.
Schwann cells have been consistently shown to be one of the most effective therapeutic cell types in transplantation and regeneration after experimental spinal cord injury (Pinzon et al., 2001; Oudega et al., 2005). These cells reduce the size of spinal cysts, remyelinate axons (Chen et al., 2009) and improve functional recovery in spinal cord injury (Biermaaskie et al., 2007). They have also been shown to produce number of growth factors that initiate and support the growth of axons, and that these cells express cell adhesion molecules on their surface for axon guidance (Tabesh et al., 2009).

GFAP antibodies did stain cells in the core area. The staining was seen to be in small clusters of cells suggesting that reactive astrocytes did infiltrate the core. In many cells, GFAP staining co-localized with S-100 staining, indicating that the cells were Schwann cells. High levels of GFAP in Schwann cells may differentiate them as being a non-myelinating phenotype (Jessen et al., 1990), with functional roles in the maintenance of unmyelinated (nociceptive) axons. Non-myelinating Schwann cells are involved in the formation of Remak bundles, wrapping and separating unmyelinated axons by means of thin cytoplasmic extensions (Corfas et al., 2004).

The proportion of channel area occupied by S-100 positive staining was not statistically different across the 3 animal groups. This was a surprising result in that one would expect the proportion of Schwann cells in the SC animal group to be the highest at 4 weeks given scaffold loading with that cell type. The presence of similar numbers of Schwann cells in MSC and Matrigel channels as in the dedicated SC scaffold supports the idea that (i) the majority of S-100 cells seen must have migrated from the cord environment, and (ii) there must be a process of transition regarding the cells we implanted, such that they perhaps migrated from the scaffold, or died. That a high proportion of implanted Schwann cells in fact die within the transplant environment has been demonstrated by the Bunge group in Miami using a contusion model (Pearse et al., 2007). The group developed two labelling techniques to identify transplanted cells, using in situ hybridization against a Y chromosome (male cell engraftment into a female animal), or GFP delivered from a lentiviral vector (female cell engraftment into a female animal). Using the Y chromosome label, the number of Schwann cells present within the injury epicentre was quantified stereologically. At 3 days post
implanation, 82.6 +/- 12.4 % of the original cell number (1 x 10^6 cells) were seen to have survived. At 3 weeks post transplantation the number fell to 22.4 +/- 4.3 %. At nine weeks the number was unchanged. Using the GFP label, surviving cell and cell loss was equivalent to that seen with the Y chromosome probe. No migration of labelled cells was observed into regions of dense GFAP immunoreactivity in the injured area or into the normal cord white or gray matter.

The study was also able to distinguish between the labelled transplanted Schwann cells and endogenous non-myelinating Schwann cells using antibodies against the low affinity neurotrophin receptor p75. In injured but not transplanted animals, p75 positive cells were observed within the injury site and dorsal column trails at the early 3 day mark and increased over time at three and nine weeks. In injured and transplanted animals, the numbers of p75 and GFP positive were equivalent, given the proportion of transplanted cells that would also express p75. However a transition of cell markers was observed over time, such that at 3 and 9 weeks p75+ cell counts were significantly higher than counts of GFP cells, 301% higher at 3 weeks, and 307% higher at 9 weeks. To account for cell loss, several mechanisms were proposed including the robust immune response (Laylor et al., 2005), poor vascularisation and gas exchange (low availability of oxygen and acidity with carbon dioxide accumulation), (Griffiths, 1976), and production of cytotoxic nitrite and lipid free radicals within the anoxic environment (Braughler and Hall, 1992; Bao and Liu, 2002). Cell stress given the transition from a nutrient rich culture into the hostile injury environment is also in evidence (Brundin et al., 2000). While the numbers of cells dying approached 80 % in a contusion model, we would anticipate even higher rates of death in a scaffold model, given that the environment would probably be even more anoxic and/or immune stimulating than that within the intact, albeit injured cord.

Further work has shown that a transition occurs between mature and immature non-myelinating phenotypes in Schwann cells that migrate from the peripheral roots. (Nagoshi et al., 2011) used a complete transection model in transgenic mice whereby eGFP is expressed only in cells of the neural crest lineage. After 7 days post transection, eGFP+ Schwann cells were seen to have migrated into the lesion from the peripheral nerve, following which time a phenotypic dedifferentiation occurred. The phenotype changed from a mature P0+ mature myelinating cell, to a predominance of P0-/p75+ phenotype that was then
seen to proliferate within the lesion. Dedifferentiation was corroborated by increased cellular expression of phosphorylated c-jun, which is an inhibitor of myelin associated genes in peripheral nerve. Subsequent to the 7 day time point, cells reacquired their maturity (P0+) over the course of time points at days 14, 28 and 56.

In our model, the end result was an equivalent degree of Schwann cell migration across the three animal groups given the common restraints of the gliotic environment. If GFAP/S-100 positive cells in the scaffold channels do represent an immature non-myelinating phenotype, (such as that seen with p75 staining (Plant et al., 2002)) this would further support the idea they were derived from the animal. We did not stain the Schwann cells with GFAP prior to implantation as part of their characterization. However a non-myelinating phenotype suggests adaptation by innate cells to an environment in which non-myelinated axons are extending. A low proportion of channel area (less than 10%) was occupied by the double labelled cell type. Under normal circumstances, Schwann cell are inhibited from migrating into the central nervous system by astrocytes. In vivo (Baron-Van Evercooren et al., 1992) and in vitro (Afshari et al.), Schwann cells are actively excluded from areas occupied by astrocytes. (Lakatos et al., 2000) showed astrocytes and SCs segregated into non-overlapping groups in culture, that astrocytes hypertrophied when in contact with SCs, and that the number of astrocytes expressing chondroitin sulfate proteoglycans was increased in the presence of SCs in co-culture. There is evidence that the presence of Schwann cells might induce astrocytes towards an injury response, including reactive hypertrophy, boundary formation, and increased GFAP and CSPG expression. Further studies showed that Schwann cell conditioned media was sufficient to induce the response, implicating a secreted factor, likely to be a fibroblast-growth factor family member on the basis of FGF ligand and receptor antibody studies (Santos-Silva et al., 2007).

The same study also implicated heparin sulfate proteoglycans as modulators of the response. When transplanted into the spinal cord, sharp boundaries between astrocytes and transplanted Schwann cells have been observed, showing abrupt transitions between the graft and host astrocytes (Afshari et al.). In the study cited, Professor Fawcett’s group has shown that aggregan produced by astrocytes inhibits Schwann cell motility on monolayers
and that proteoglycan knockdown by RNA interference or glycosaminoglycan side chain digestion improves Schwann cell migration. In our study, sharply demarcated transitions between central core and the peripheral zone were characteristic of the Schwann cell channels. There was little intermingling of Schwann cell and astrocytes seen, and axonal regeneration was limited to the areas where Schwann cells were present.

Schwann cells have long been known to more freely migrate into the injured cord however (Feigin and Ogata, 1971) with breakdown of the normal cord structure. Here the cells may contribute to healing by associating with injured axons and remyelinating fibres within the lesion (Takami et al., 2002). In contusion injuries, ‘endogenous repair’ was noted, in that the injury cavities often became partially filled with nerve fibers and associated Schwann cells, and that the amount of fiber and Schwann cell ingrowth was inversely related to the severity of injury (Beattie et al., 1997). Most fibres originated from the dorsal root. In addition, ependymal cells from the area of the central canal were seen to develop a trabecular network which may serve as repair scaffolding. In a model of ethidium bromide induced spinal cord injury, dorsal columns remyelinated by endogenous Schwann cells had even formed Ranvier Nodes with the normal patterns of voltage gated sodium and potassium channels (Black et al., 2006) at one year post injury. Recent work has focused on the genetic modification of Schwann cells to facilitate their migration and functional integration into the astrocytic environment of the injured cord (Lavdas et al., 2008). The study shows that Schwann cells can overcome the inhibitory effects of aggegran on monolayers by genetic upregulation of surface integrins. (Afshari et al.) have further characterized the molecular interactions involved in limiting migration. Astrocytes produce EphrinA ligands, which bind EphA receptors on Schwann cells, in turn signalling for reduced integrin function via phosphorylation of the oncogene guanine nucleotide exchange factor pVAV2. Blocking antibodies against the EphA4 receptor on Schwann cell and introducing siRNA targeted against VAV2 can both restore the capacity for Schwann cell migration on astrocytes in culture.

In vivo, Schwann cells genetically modified with retrovirus to overexpress the cell adhesion molecule L1 were grafted rostral to a compression injury in mice. Up to a time point of 3 weeks, but not thereafter, mice demonstrated accelerated functional recovery correlated with enhanced myelination and
increase serotonergic fiber sprouting in the L1-overexpressing group (Lavdas et al., 2009; Lavdas et al.). Similarly the presence of Schwann cells within OPF+ scaffolds at the time of injury may pre-condition the cord for improved regeneration and remyelination as the cells do not need to migrate in the early phases of injury.

If Schwann cells are derived, even in SC-loaded channels, from the local interfaces of the CNS and PNS, and if they associate with regenerated axons, it is possible that many of the axons seen within the channel are themselves from a peripheral nerve root. In that case these fibers may not be helpful in rejoining central pathways within the cord, but may instead form peripheral arcs, particularly those of nociception. The origin of regenerated axons, and Schwann cell-axon interaction was an issue that the laboratory tried to address early in the development of PLGA scaffolds. That Schwann cells interact directly with axons within PLGA scaffolds, and that they actively myelinate the axons, was previously been demonstrated by (Chen et al., 2009). Electron microscopy showed myelinated axons in the transverse sections of the implanted PLGA scaffolds 2 months after implantation, with a pattern and extracellular matrix constituent with Schwann cell myelination. Fast blue tracing studies showed the origin of many cell bodies to be in the ventral horn region, with bidirectional extension through the scaffold. Fast-Blue studies are planned to be done in charged OPF scaffolds containing neurotrophic Schwann cells.

As axonal regeneration is most successful in our model in a setting where Schwann cells are used in the channel, it will be important to understand the interactions between Schwann cells, astrocytes and nerve regeneration in the setting of injury. The model of transection injury and scaffold placement we describe has good potential as a means to isolate and control the variables in such investigation. From the data presented here, we are proposing that axonal regeneration within charged OPF scaffolds is supported best by Schwann cells, and that there is an interaction of CNS (reactive astrocytes) and PNS (Schwann cell) cellular elements to create a hybrid environment. The regenerating core environment by default has elements recruited in from the peripheral nerve system, as seen in Matrigel scaffolds, consistent with what occurs naturally in the human cord following injury. Implanting Schwann cells may pre-condition the environment to improve axonal extension. Augmentation of the peripheral
elements by pre-loading scaffolds with Schwann cells improves the efficiency of axonal growth, despite the degree of transplanted cell death.

**The influence of MSCs on the regenerative core**

Caplan and Dennis have described MSCs as having two distinct functions: to provide for replacement units for expired cells in mesenchymal tissues, and to have trophic effects on cells in their vicinity by the secretion of functionally active agents, without themselves becoming newly differentiated phenotypes (Caplan and Dennis, 2006). When introduced into the CNS, it is proposed that MSCs do not differentiate into neurons or neural support cells such as astrocytes. This is in keeping with our observations in this study and in associated work within the lab (Rooney, 2007; Rooney et al., 2009). MSCs have been shown to secrete vascular endothelial growth factor, nerve growth factor, and brain-derived growth factor (Chopp and Li, 2002). In the context of CNS healing, MSCs may supply secreted agents for the inhibition of scar formation and apoptosis (Chen et al., 2003), increase angiogenesis, or to stimulate regeneration of the environment via glial-axonal remodeling (Li et al., 2006). Neurogenesis and neurogenesis and synaptogenesis may occur from resident neural stem cells (Li et al., 2001) that are stimulated factors secreted by MSCs. In other therapeutic applications such as meniscus repair or cardiac muscle regeneration, MSC trophism is likely to be of greater significance than the differentiation of the cell into chondrocytes or myocardium in situ (Caplan, 2009).

In our study OPF+ scaffolds pre-loaded with eGFP-MSCs did not support axonal growth to any substantial degree by the 4 week time point. The implanted cells in the MSC groups do survive in the scaffold and can be seen in albeit very sparse numbers within the channel core area by eGFP fluorescence at 4 weeks (Figure 3.14). MSC cell death following implantation is likely to be on the same order as that seen in Schwann cells within the scaffold, given the same hostile influences are at play. The planned time course experiments will further detail the degree and rates of cell loss. However, as seen in the Schwann cell group, preimplantation of MSCs within the scaffold resulted in a defined tissue architecture which was clearly distinguishable from the other two groups.

That the tissue within MSC-scaffold channels contained virtually no regenerated axons was a result that conflicted with the majority of studies using
MSC transplantation techniques in spinal cord injury. By means of trophic effects, tissue sparing via immunomodulation, and tissue repair, MSCs in spinal cord injury have been shown to enhance axonal regeneration and promote functional recovery in animal models. Since (Chopp et al., 2000) first described MSC engraftment into the injured cord with expression of the NeuN marker and improved functional BBB scores, 17 other in vivo animal studies have followed. The current understanding has been recently and expertly reviewed (Wright et al., 2011), specifying the type of spinal cord lesion, in what way the MSCs were transplanted, the histologic outcome, and the functional outcome of the animals. The majority of studies have combined a weight-impactor contusion injury in the rat thoracic followed by direct injection of cultured allogenic MSC into the substance of the cord lesion (Chopp et al., 2000; Hofstetter et al., 2002; Wu et al., 2003; Ankeny et al., 2004) either at the time of the injury or within 7 days. (Himes et al., 2006) injected cells into the lesion or into the rostral and caudal tissue adjacent to it in graded contusion injuries. In one series (Vaquero et al., 2006), cell injection was delayed until 3 months post injury and the animals were followed out to 1 year (Zurita and Vaquero, 2006). The studies also compared direct injection into the lesion with intravenous infusion of equivalent cell numbers. One group used cell injection into the fourth cerebral ventrical following contusion injury (Ohta et al., 2004).

Focal demyelination of the thoracic cord by either x-irradiation or ethidium bromide was followed by intra-lesion injection (Akiyama et al., 2002) and intravenous injection (Akiyama et al., 2002) with evidence of a peripheral pattern of remyelination seen. In the cervical cord, axonal regeneration through a microwire lesion in response to direct injection of MSCs (Lu et al., 2005) was compared to BDNF-MSCs and to neurally differentiated MSCs, which were seen to lose their neuronal phenotype upon implantation. (Paul et al., 2009) compared delivery methods via direct lesion injection, intrathecal infusion or intravenous infusion after cervical cord hemisection. In all cases, the number of cells injected ranged from $2.5 \times 10^5$ to $3 \times 10^6$. Histologic parameters have included successful MSC engraftment, remyelination, reduced cavity formation, cell bundle or bridge formation facilitating axonal regeneration, and enhanced Schwann cell migration into the wound. Where functional assessments were done, outcomes included
statistical significance of improved BBB scores, increased axonal conduction velocity, exploratory rearing behaviour, and sensory sensitivity to thermal stimuli.

In regards to polymeric deliver of MSCs, (Sykova et al., 2006) have used methylacrylate derivatives to seed MSCs into a cord hemisection with reduction in lesion size noted, higher scores in BBB testing than did control animals and also showed a faster recovery of sensitivity in their hind limbs using the plantar test. These seeded scaffolds were more recently used in bridging a chronic injury, whereby the scaffold was implanted 5 weeks after a balloon compression injury (Hejl et al., 2010) and behavioural testing up to 6 months post implantation demonstrating functional improvements. In this study hydrogels were infiltrated with axons which were myelinated with Schwann cells. Blood vessels and astrocytes also grew inside the implant. MSCs were reported as present in the hydrogels 5 months after implantation.

Our studies, here and (Rooney et al., 2011) are among the very few studies that have used a complete transection model for the polymeric delivery of MSCs. (Zeng et al., 2011) have used a collagen-gelatin sponge covered by a thin film of PLGA and seeded with MSCs for implantation into transection model. No mention is made whatsoever of axons regenerating into the graft, and rather the study remarks upon fibronectin deposition and angiogenesis. Our results may demonstrate the necessity for MSCs to interact with intact nervous tissue such as would be available in close proximity to the injured area in a contusion or even hemisection model. By cell loading and scaffold volume estimates, we are delivering on the order of $2.38 \times 10^5$ MSCs into the cord environment, which is a lower cell number than any of the studies cited above. In completely transecting the cord, and in implanting MSCs in relative isolation to intact tissue, we propose that MSCs are unable to exert sufficient effects for robust axonal regeneration after 4 weeks in the animal.

**The relationship between axon regeneration and channel vasculature**

MSC channels may not have been able to support axon growth, but robust angiogenesis was the striking feature in their tissue architecture. The REMEDI group (Duffy et al., 2009), in the context of myocardial repair, has shown MSCs to be capable of promoting angiogenesis in vitro. (Wu et al., 2007) showed improved granulation tissue formation and angiogenesis in a skin excision wound
healing model in mice. Here, MSC conditioned media promoted the organization of endothelia cell tube formation in vitro in human umbilical vein endothelial cells (HUVEC). Using RT-PCR and western blot analysis, higher amounts of vascular endothelial growth factor and angiopoietin-1 in MSCs were detected in culture following hypoxia than in control fibroblasts, as well as in MSC-treated wound extracts. Measurements have also been made with an in vitro assay using human brain endothelial cells, in which supernatants from MSC cultures were shown to induce the rapid formation of tubules, and MSCs implanted into the vascular rat cornea created new vessel networks (Hamano et al., 2000). In (Zeng et al., 2011) this study also demonstrates a higher proportion of von Willebrand staining in MSC grafts at the host-implant interface, and suggest VEGF staining is enhanced in MSCs adjacent to the vessel. Marrow cells may also contribute to VEGF-induced angiogenesis by supplying matrix metalloproteinase-9 (Hao et al.) allowing for the clearance of matrix which may be impeding vessel growth.

We recently published observations of vessel formation within OPF+ scaffold channels loaded with eGFP-MSCs at 4 weeks (Rooney et al., 2011). It is difficult on H&E and Masson’s Trichrome staining to distinguish which open, lined spaced represent cystic structures or vessels, unless the vessels are large enough to have a fully developed endothelium that can be clearly recognized. In this study, the blood vessel endothelia was visualized in channels of eGFP-MSC, SC and Matrigel scaffolds with antibodies directed against von Willebrand factor using a TRITC secondary. Significantly higher numbers of vessels (45.67 +/- 3.05 capillaries per scaffold section) were observed in the eGFP-MSC group than that seen in the SC (27.25 +/- 3.45) and control (11.58 +/- 1.94), p< 0.001.

To extend these studies, and to try to understand if there was any relationship between vascular formation in scaffold and the tissue's capacity to support axons, a detailed analysis of vessel architecture was done using unbiased stereology. Volume fraction (Vv), length density (Lv) and surface density (Sv) for the channel vasculature of each group were estimated and the values applied to the mean channel volume to give estimates of physiologic reference in the animals. The fraction estimates were also used to calculate other physiologic valves, mean vessel diameter, mean vessel cross-sectional area and the radial diffusion distances of channel vessels. By all measures the Matrigel channels, which minimally supported axonal growth, were relatively avascular compared to
Schwann cell and MSC channels, with reduced Vv, Lv, Sv. However Matrigel vessel architecture was similar to Schwann cell channels in that the mean vessel diameter and cross-sectional area were comparable. Where Matrigel fell behind was in the number of vessels and consequently the length density, or the total estimated vessel length in the section, was low. MSC channels did not support axonal growth despite being, at least on the surface, highly vascular. Very large vessels formed in MSC tissue, and consequently the Vv and blood volume were high given that the vessels had a much larger diameter and cross-sectional area than the other two groups. However, the blood flow rate through MSC vessels would be consequently reduced given the importance of vessel radius exponentials on flow. While the MSC vessels were large, comparably there were not many of them.

By sheer weight of numbers contributing to high total vessel lengths (over 1 mm in total per channel section), Schwann cell channel vessels were also able to maintain comparable volume and surface area estimates to MSC channels. A multitude of small diameter vessels in Schwann cell channels was observed, having tight mean cross sectional areas, with consequently higher rates of flow, delivering as much blood volume as MSC vessels. That the primary fractional estimates might correlate significantly to axon number was confirmed in an analysis that included both Matrigel and Schwann cell channel to provide sufficient variability for the analysis. Highly significant correlations were seen between increasing axon number and increasing length volume, surface density and volume fraction. A significant correlation also was shown between decreasing vessel diameter and increasing axon numbers, implicating the importance of adequate blood flow rates to axonal regeneration.

The tightest correlation seen (Spearman r = 0.7852, p<0.0001) was between axon number and the length volume. The Lv is of particular physiologic importance as it relates to the radial diffusion distance, the measure of a cylindrical space of tissue around the vessel that will be influenced by diffusion from it. Of the three channel types, Matrigel demonstrated the highest radial diffusion distances (up to 800 µm) followed by MSC vessels (600 µm). Vessels in Schwann cell channels had distances of around 200 µm. The estimates show that when there is insufficient vessel length available, the tissue must rely on distant vessels for blood support, and thus must be comparatively hypoxic. The radial
diffusion distances in human spinal cord grey and white matter have been calculated in tissue sections obtained from 4 cadaver donors (Dockery and Fraher, 2007). The values were between 15 and 20 µm in both regions, slightly higher in white matter than grey, and importantly were seen to be highly conserved across the 4 individuals. The coefficient of variance ranged between 3.3 and 5.6%. Conservation of neurovascular parameters in turn applies something of importance for species evolution and survival. Here we have shown the relationship between radial diffusion distances to correlate significantly with axon numbers as a hyperbolic function. In the setting of tissue engineering we have insight into variable axon and vessel numbers in regenerated tissue, from experiments that worked well and those that did not, in addition to an analysis of the final product in the normal animal. From our data we show the necessity of improving overall vessel length densities in tissue-engineered spinal cord in order to achieve physiologic levels of both vessel and axon numbers.

**MSCs and immunomodulation in the channel core**

The scaffold channels were seen to be a highly inflammatory environment in each animal group, with dense infiltrates of CD45+ B cells, microglia expressing Iba1 and CD3+ T cells. Whereas the distribution of B-cells was throughout the channel area, CD3+ cells, and to a more concentrated degree, microglial cells populated the core compartment of scaffold channels. Microglial cells were also seen to participate in the multinucleated cell types formed at the interface between channel tissue and the polymer wall. In comparing the volume fraction of each cell type as a proportion to channel area, Schwann cell channels had a significantly less proportional volume of B-cell and T-cell infiltrate over Matrigel control, but had a higher proportion of microglia in the channel than the other groups, localized to the regenerative compartment. MSC channels demonstrated a significantly higher proportion of B cells and microglia than control, but significantly T-cell infiltration over the control group suggesting a immunomodulary role in relation to T-cells.

An important aspect of the therapeutic potential of MSCs is their ability to modify the immune environment an area of injury, to dampen the immune response avoiding excessive damage to the areas being healed. Primarily, MSC immunosuppressive interactions are both directly and indirectly with T-cells.
MSCs share surface markers with thymic epithelium and express adhesion molecules essential for T-cell modulation. MSCs express MHC class I on their surface, and may have stores of class II intracellularly, but lack surface MHC II and the corresponding co-stimulatory molecules required for T-lymphocyte activation (CD80, CD86 or CD40) even after they have been stimulated with interferon-γ (INF-γ) (Le Blanc and Ringden, 2007). Early studies described in vitro that MSCs were able to suppress naïve and memory T lymphocyte activation and proliferation in response to alloantigens and nonspecific mitogens (Di Nicola et al., 2002). A lack of T-cell response occurred even in the presence of other antigen presenting cells or stimulatory antibodies to T-cell CD28, CD80 or CD86. (Le Blanc et al., 2003). The end effect is the inhibition of T-cell activation, rather than inducing apoptosis, as measured by a lack of cellular proliferation, no increase in INF-γ secretion by T-cells, and a decrease in markers associated with T-cell activation such as CD25, CD38 and CD69 (Klyushnenkova et al., 2005). The suppressive effect is also demonstrated by MSCs which have been differentiated to osteocyte, adipocyte and chondrocyte lineages. (Le Blanc et al., 2003).

With regard to CD8+ cells, MSCs themselves evade cell lysis mediated by cytotoxic T-cells (Ryan et al., 2005), and suppress their activity in destroying other target cells, provided they are present prior to the activation of the cytotoxic phase (Rasmusson et al., 2003). A similar situation applies to natural killer cells, whereby MSCs are able to suppress proliferation prior to activation, but only at high numbers not applicable to use in vivo, and were not able to suppress lysis by activated cells (Spaggiari et al., 2006).

MSCs are also thought to mediate a shift in CD4+ T-helper cell types, suppressing Th1 by downregulation of INF-γ and interleukin-5 (IL-5), and augmenting Th2 cell development via IL-4 production. Th1 cells are involved in the activation of naïve T-cells into INF-γ secreting cells, as well as assisting cytotoxic cells, thus proliferating the overall inflammatory environment. Th2 cells are potent immunoregulator cells, induced by increased levels of interleukin-10 (IL-10) (Kode et al., 2009). MSCs also affect T-cell activation by inhibiting the formation of mature dendritic antigen presenting cells from their monocyte precursors. Mature cells dendritic cells in the presence of MSCs have a reduced
potential to activate CD4+ cells, through inhibition of their own INF-γ, IL-12 and TNF-α secretion (Jiang et al., 2005), and downregulation of co-stimulatory molecules on their surface (Zhang et al., 2004).

MSC effect upon B-cells is less clear, and seems to be dependent upon the ratio of cells used in the assay. The proliferation response to antigen, such as streptococcus protein A, and stimulatory antibodies to CD-40 or IL-4, was inhibited by MSCs, whereby the cells were seen to arrest in the G0G1 cell cycle phase (Corcione et al., 2006). B-cell migration to chemotaxis might also be affected by MSCs, in that a reduced expression of CXCR4/5 and CXCL13 was observed. The pattern of antibody secretion in response to MSCs appears to be dependant upon the cell-dose. Total IgM, IgG and IgA was reduced if MSCs are present at a 1:1 ratio, but IgG was increased in different assay systems at lower cell doses (Le Blanc and Ringden, 2007).

MSCs appear to regulate the immune environment by ligand binding during cell-cell contact surveillance, and by the secretion of soluble factors. T-cells activation is inhibited by MSC-derived prostaglandin E2 via the induction of regulatory T-cells. The presence of T-cells will induce COX-2 enzyme activity and prostaglandin production in MSCs (Aggarwal and Pittenger, 2005). While much seems to develop upon elaboration of the inflammatory response by INF-γ, MSCs create an immunosuppressive microenvironment capable of modulating alloresponsiveness even in the presence of the cytokine. Recent work at NUI Maynooth demonstrated that INF-γ could not blunt MSC inhibition of lymphocyte proliferation, but resulted in the upregulation of inhibitors such as Indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor, (HGF) and transforming growth factor β1 (TGF-β1) (Ryan et al., 2007). MSCs in this model were seen to express cyclooxygenase 1 and 2 and produce prostaglandin E2 constitutively, and use of indomethacin reduced allo-suppression. IDO is secreted by MSCs in response to INF-γ and inhibits T-cell proliferation by means of tryptophan metabolite accumulation (Munn et al., 1998; Ryan et al., 2007). Further use of tryptophan antagonists restored proliferation in response to INF-γ, while use of the catabolite kynurenine restored suppression. Transforming growth factor-β1, hepatocyte growth factor, IL-10 and IL-2 may also inhibit T-cell activation (Di Nicola et al., 2002). Cell contact and prostaglandin synthesis by MSCs may also
be important in inhibiting T-helper differentiation towards a T17 phenotype, a
CD4+ T-cell subset characterized by IL-17A secretion effecting adaptive and
auto-immunity (Duffy et al., 2011). IL-6, IL-8, chemokine ligand 2, nitric oxide,
HLA-G, stem cell-derived factor 1 (SDF1) and vascular endothelium growth
factor (VEGF) are also implicated, as the complexity of our understanding
mounts.

**MSCs as pericytes and granulation tissue formation**

Much of the recent work done regarding MSCs-induced angiogenesis has
been done in the context of skin dermis remodeling. Unique subpopulations of
what were then thought to be dermal fibroblasts were seen to be located in the
superficial dermis and contributed to the organization and maintenance of the
microvasculature (Sorrell et al., 2008). MSCs were then observed to support the
development and stabilization of vascular tube-like structures in vitro, co-aligning
with them to increase their number and complexity (Sorrell et al., 2009). In a
landmark study, (Crisan et al., 2008) identified two populations of cells which
were developmentally and anatomically related to blood vessel wall in muscle and
in other fetal and adult organs, by means of molecular markers and purification by
flow cytometry. The cells were identified as skeletal muscle myoendothelial cells
co-expressing endothelial and myogenic markers, and pericytes, or mural cells,
surrounding capillaries and microvessels. Both cell types demonstrated
multilineage differentiation into skeletal myofibres, bone, cartilage and adipocytes, the hallmark of MSC characterization or identity. Pericytes from
various adult and fetal tissues have also shown expression of classically MSC-
type markers, CD44, CD73, CD90 and CD105 (Augello et al., 2010). It is notable
that we used three of these markers to characterize our own implanted MSCs.

Pericytes, also known as mural or Rouget cells, are located in vivo in close
connection to the endothelial cells of arterioles, capillaries and venules, where
they are thought to function in vessel stabilization, synthesis of matrix proteins,
have macrophage-like properties, and be active in immunologic defences (Diaz-
Flores et al., 2009). Thirty years ago it was suggested that pericytes might be
progenitors for adipocytes during tissue injury (Richardson et al., 1982). Twenty
years ago the cell type was proposed to be a supplemental source of osteoblasts in
periosteal osteogenesis (Diaz-Flores et al., 1992), and Monoastral Blue staining
provided evidence that pericytes were involved in generating cartilage and bone (Diaz-Flores et al., 1991). Other stem cells may inhabit the perivascular niche as well, as evidenced by the anatomical location of hematopoietic (Kiel et al., 2005) and neural stem cells (Dore-Duffy et al., 2006) in the CNS microvasculature. Populations of pericytes may in addition have a neuroectodermal origin, derived from Sox1+ neuroepithelial/neurocrest cells (Takashima et al., 2007), an observation that may explain the presence of nerve growth factor receptors on bone marrow MSCs, and that MSCs can have potential for neurodifferentiation. The observation might also explain the tolerance the CNS environment might have towards mesenchymal cell transplantation, where the cells remain resident and viable for prolonged periods of time. Pericytes exist in the brain and retina (Frank et al., 1987), at higher density than in any other organ (Shepro and Morel, 1993). Here again they are thought to function in the stabilization and maturation of the neurovascular system as well as the blood brain barrier. Brain pericytes also have been shown to possess pluripotential activity, and are recruited at times of ischaemic injury (Kamouchi et al., 2011). There remains much interest in MSC infusion following stroke injury since the early studies (Chopp and Li, 2002) showing enhanced repair. That brain pericytes can contribute to CNS regeneration is in evidence, where the cell type gave rise to neurons and glial cells in the subgranular zone of the dentate gyrus in monkeys following ischemia (Yamashima et al., 2004). Also in a model of stroke, immature neurons closely associated with the remodeling vasculature, with neurogenesis thought to arise from GFAP-positive progenitor cells in the subventricular zone. Neurogenesis and angiogenesis may be linked through vascular production of stromal-derived factor 1 (SDF1) and angiopoietin 1 (Ang1). These factors can promote neuroblast migration and behavioral recovery (Ohab et al., 2006).

The current understanding based on the evidence is that MSCs most likely represent subpopulations of pericytes (Caplan, 2008), a concept that connects MSCs to immune and vascular functions implicated in tissue repair via granulation tissue formation (da Silva Meirelles et al., 2008). da Silva Meirelles proposes the model that MSCs are situated throughout the body as pericytes, and that MSCs isolated from bone marrow, adipose tissue or elsewhere are derived from the blood vessel walls. This conclusion is based upon a comprehensive review of MSC surface markers and those they share with both pericytes, vascular
smooth muscle cells and mature endothelium, as well as the plasticity of pericytes types to form other lineages. The pericyte/MSC is thought to participate in a classic pathologic transition of wound healing involving granulation tissue intermediates. The cell is liberated with disruption of blood vessel integrity, and subsequently migrates into the injured area. The cell transitions in a role from tissue and immune homeostasis in the normal vessel, to injury response in the setting of acute inflammation, hypoxia and blood hemostasis. Through the development of intermediate granulation tissue, pericyte/MSC proliferate and begin to exert immunomodulatory and trophic effects. Within granulation tissue, pericytes secrete bioactive factors to protect from B and T-cell mediated destruction, repair the injured tissue by limiting scarring and apoptosis, inducing angiogenesis and producing mitogens to stimulate the local progenitor cells. Maturation of tissue remodeling can occur with restoration of blood vessel integrity, situation of the pericyte back to its vascular location to stabilized vessel formation. Pericytes left peripheral to the vessel may undergo apoptosis or remain in situ as progenitor cells residing between cells specific to the tissue being healed (da Silva Meirelles et al., 2008).

The hallmarks of granulation tissue and tissue repair are the recruitment of macrophages, proliferation of small vessels (endothelium and pericytes) and proliferation of fibroblasts-myofibroblasts. Granulation tissue is a provisional tissue type in the repair process from which there can be reconstruction of the original tissue stroma, or replacement of the original stroma by permanent non-specialized fibrous scarring. The perivascular space, with pericytes, perivascular fibroblasts, and homing cells from the bone marrow, is thought to be the niche for progenitor cells critical to repair through granulation (Diaz-Flores et al., 2009). Moreover the granulation tissue itself probably represents a “paracrine transitional organ,” as termed by the authors, mediating healing from the acutely injured to the remodelled state, through the interaction of its local progenitor pericytes, recruited circulating bone marrow cells and stem cells that are resident to the original tissue. In tissue engineering, the interactions between regenerative elements and the components of granulation tissue may be essential, and may play a role in programming of the final tissue structure.

The introduction of cultured MSC into a wound, CNS or elsewhere, would be the equivalent of establishing tissue repair at the granulation phase. Cell
migration is not necessary because the cells are placed directly into that environment and as such the process is accelerated. Cultured MSCs can be directly delivered to damaged areas in the spinal cord in large numbers. We have presented here several observations that MSCs may be playing such a role in our scaffold model. Primarily, the tissue type has a histopathologic appearance of granulation tissue, based on expert opinion. MSC channels have a distinct vascularity comprised of large calibre vessels secondary to induced angiogenesis. eGFP positive cells in MSC scaffold channels are located in distinctly perivascular locations related to new vessel formation. In Vimentin staining of MSC channels, cells fluorescing in the FITC wavelength, which must be either eGFP or vimentin positive cells, are either circumferentially perivascular or have integrated into the vessel wall itself. Pericytes are thought to be the precursor source of fibroblasts within granulation tissue (Diaz-Flores et al., 2009). We do not observe GFAP positive cells having involvement in vessel structures, and it is unlikely that an astrocyte, albeit immature would exclusively express Vimentin. We have also observed that inflammatory environment is dampened in MSC channels over control channels particularly in the proportion of T-cell infiltrate.

In short, we propose that MSCs/pericytes transplanted into the CNS are doing what they are supposed to do, in the context of regenerating tissue via provisional or protoplasmic tissue intermediates. To what extent such granulation tissue will support axonal regeneration and spinal cord remodelling is not yet clear. Whether this tissue is of a type usually formed in CNS healing is not known. It may do so, in ways that we have not yet looked for, and tissue engineering strategies are augmenting the process through hybrid tissue formation. At least at the 4 week time interval this tissue type does not support axon growth. Where MSCs in other studies contribute to regeneration, the difference may lie in their integration into host spinal cord tissue being injected as a cell suspension, as opposed to being presented en bloc as a tissue entity in and of itself as we have done. If given more time in the animal, we can speculate that the granulation tissue intermediate in scaffolds may be a useful and perhaps necessary adjunct to axonal growth supported by other factors such as Schwann cells. It may be necessary to take the spinal cord through the transition of healing mediated by pericytes and granulation tissue to form a transitional vascular bed of tissue. Scaffolds filled with a combination of Schwann and MSC types may be
seen to augment the numbers of regenerating axons in the central core beyond the capacity shown consistently by Schwann alone. This may confer an advantage in using the cell for its own innate paracrine and autocrine function, or following genetic modification, as a means to deliver neurotrophic factors and the like. In the following chapter we discuss the development of Schwann cell and MSC lines genetically modified by retrovirus to secrete functional neurotrophins. Conversely perhaps the tissue will go by way of permanent fibrosis, and in that case I would anticipate the scar tissue would be of a mesenchymal or fibroblastic pathologic appearance resident in the CNS and not one of gliosis.
Chapter 4:
Retroviral Vector and Neurotrophic Cell Line Development

4.1 Introduction

4.1.1 Neurotrophin Delivery and the Promise of Regional Control

Polymer scaffolds offer the means to deliver neurotrophic molecules to the injured spinal cord to improve axonal regeneration. A common fabrication technique is to incorporate a neurotrophic factor into the polymer mix prior to polymerization, whereby the entire scaffold elutes the drug in an isotropic fashion. Natural materials are particularly well suited to this approach, given their polymerization rarely involves processes that will denature or destroy the factor. This approach was among the earliest used in the spinal cord, whereby a collagen matrix with NT-3 was placed into a transection model (Houweling et al., 1998). The collagen construct specifically attracted corticospinal sensory tracts, promoting partial recovery, and in doing so exemplified important principles of polymer scaffold design. The chemotrophic effect of neurotrophins locally delivered from polymers enhanced the regenerative capacity of the scaffold, and did so in a manner that can be regionally controlled on the basis of the neurotrophin’s target axonal population.

A second delivery approach is to use synthetic multichannel channel scaffolds whose conduits are filled with eluting polymer. Iannotti et al (2003) filled a minichannel PAN/PVC with Matrigel containing recombinant glial cell derived neurotrophic factor (GDNF), or GDNF Matrigel with additional Schwann cells (Iannotti et al., 2003). GDNF attracted axons primarily of propriospinal phenotype, and the effect of Schwann cells was synergistic. The polymer matrix in the channel itself can also significantly impact the axonal phenotype regenerated. When filled with either Matrigel, collagen, fibrin, or methylcellulose, fibrin matrix within pHEMA-MMA channels demonstrated preferential regeneration of descending reticular neurons, while methylcellulose promoted growth of vestibular and red nucleus neurons (Tsai et al., 2006).

Thirdly, genetically modified cells that over-express neurotrophic factors have been placed in the injured cord. Initial work, and the vast majority of work to
date, involves the injection-encephalization of cell suspensions with or without combinations of ‘cell-bridges’ or cell suspension matrices. Early on, fibroblasts and Schwann cells were transfected with retrovirus to express NT-3 (Grill et al., 1997), NGF (Grill et al., 1997) and BDNF (Menei et al., 1998) respectively. In these 3 studies, NT-3 cells supported corticospinal tract growth in a bilateral dorsal column transection model. NGF secreting cells engrafted by the same group in the same model supported dorsolateral primary sensory axons and cerulospinal axons, but did not support the growth of corticospinal or motor axons. Suspensions of BDNF-Schwann cells implanted into a complete transection whose ends were re-apposed, enhanced the growth primarily of reticular and raphe neurons.

Lentivirus was used to transfect Schwann cells to secrete a bifunctional neurotrophic protein (D15A) with NT-3 and BDNF functionality, but cell survival was poor in a PLA scaffold. BDNF-secreting MSCs in templated agarose scaffolds survived to significantly improve regeneration over that supported by the scaffold alone (Stokols et al., 2006). There is a scarcity of work at present, but in our opinion tremendous potential, to use complex topographic designs of scaffold delivery to align specific neurotropic delivery systems to corresponding axonal groups within the same scaffold framework (see Figure 1.9A). From the initial studies cited above, a great deal of work has clarified the sensitivity of select axons to specific neurotrophic support, reviewed in (Lu and Tuszynski, 2008). NGF is involved in the growth of nociceptive cells, BDNF those that modify the activity of motor neurons, NT-3 the corticospinal tracts and dorsal sensory axons, NT4/5 proprioceptive and motor modifying inputs, and GDNF the proprioceptive, dorsal sensory, and nociceptive neurons. Despite these approaches to augment neurotrophic support and phenotypic selection, there remains no convincing evidence that axonal populations are extending beyond the cellular or polymer bridges to form lasting functional connections. Furthermore, there is evidence that the inherent regenerative capacity of the axonal subtypes may be different. As recently suggested, within the spinal cord, propriospinal may exhibit a greater regenerative capacity than rubrospinal and reticulospinal, each of which is superior to corticospinal (Blesch and Tuszynski, 2009).

In this chapter, the development of a retroviral library for gene delivery of neurotrophic factors to Schwann cells and MSCs is described. Retroviral
expression plasmids encoding the cDNA transcripts for the human NT-3, BDNF, GDNF, NGF, and CNTF genes were constructed. Neurotrophin genes were cloned into the pLXSN backbone, which has been modified to contain an internal ribosomal entry site (IRES) for bicistronic expression of eGFP in target cells. DNA sequence accuracy and eGFP-neurotrophin co-expression were verified prior to the development of GP+E86 packaging cell lines for NT-3, GDNF and BDNF retroviral production. Retrovirus were titred by two methods, and are shown to produce physiologic levels of neurotrophin over a 72 hour period in target cells. Stably transduced Schwann cell and MSC lines have been made following retroviral gene transfer and cell selection for use in the OPF+ scaffolds. Conditioned media from NT-3 Schwann cells is shown to induce outgrowth from dorsal root ganglia in a dose dependent manner.

4.2 METHODS

4.2.1 Plasmid source materials

pLXSN-IRES-eGFP
The retroviral expression plasmid pLXSN-IRES-eGFP was the kind gift of Dr. Thomas Ritter at REMEDI. This plasmid was constructed from two plasmids pLXSN and pIRES2-eGFP. pLXSN is commercially available from Invitrogen (catalog number 631509, GenBank accession no.: M28248). The plasmid contains elements derived from the Moloney murine leukemia virus terminal repeat and murine sarcoma virus promotor elements designed for retroviral gene delivery and expression. The plasmid is named after designated regions of the sequence, as per convention. From 5' forward, ‘L’ designates the 5’ Moloney murine sarcoma virus long terminal repeat (LTR) containing promotor and enhancing sequences that control the expression of the gene of interest in the multiple cloning site (MCS). ‘X’ designates the \( \psi^+ \) extended viral packaging signal. ‘S’ refers to the promotor element, the early SV40. ‘N’ prefers to neomycin antibiotic selectivity cassette. A 21 base pair multiple cloning site lies just 5’ to the SV40 promotor. Upon transfection into a packaging cell line, the plasmid can transiently express, or integrate to stably express a transcript.
containing the $\psi^+$ extended viral packaging signal along with the gene of interest and selectable marker.

The plasmid pIRES2-eGFP is also commercially available from Invitrogen, (catalogue #6029-1). The construct contains an internal ribosomal entry site (IRES2) derived from the encephalomyocarditis virus (ECMV) between a multiple cloning site, and the enhanced green fluorescent protein (eGFP) coding region. The IRES sequence allows both the gene of interest within the MCS and the eGFP gene to be translated from a single bistronic mRNA. eGFP is a red-shift variant of wild type GFP (Cormack et al., 1996) optimized for brighter fluorescence and a higher-level expression in mammalian cells, excitation maximum 488 nm, emission maximum 507 nm. Chromophore mutations (Phe-64 to Leu, and Ser-65 to Thr) contribute to enhanced fluorescence (Jackson et al., 1990). The sequence was further optimized to contain 190 silent base pair changes corresponding to human codon usage preferences (Jang et al., 1988), and the gene itself is preceded by the Kozak consensus sequence to optimize initiation of translation (Kozak, 1990). The MCS in this plasmid is under the control of an immediate early cytomegalovirus promoter, pCMV.

**Figure 4.1:** Plasmid maps for the retroviral expression plasmid pLXSN and the plasmid pIRES2-EGFP.
The retroviral expression plasmid pLXSN-IRES-eGFP (Figure 4.2) was created in Dr. Ritter’s previous lab in Munich, Germany, as a new hybrid of pLSXN and pIRES-eGFP plasmids, with the excision from the latter plasmid of a 2040 bp cassette between the EcoRI site within the MSC and an Hpa1 site about 150 bp 3’ to the end of the eGFP gene. Using those two enzymes opens pLSXN at two adjacent sites in the MSC, and the pIRES-eGFP cassette was ligated in to create the new plasmid. Cutting at the EcoRI site in the pIRES-GFP brought in a number of new MCS sites into pLSXN, adding further flexibility for future cloning.

**Figure 4.2:** The retroviral expression plasmid pLSXN-IRES-eGFP was made from inserting a 2040 bp cassette containing IRES-eGFP sequences into the pLXSN backbone. A new MCS was created during the insertion (shown upper right).

**Neurotrophin plasmids**

A retroviral expression plasmid library was developed for the delivery of five neurotrophic genes to target cells, neurotrophin-3 (NT-3), brain-derived
neurotrophic factor (BDNF), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CTNF). In order to source human sequences for these genes, we utilized the website GeneCards, developed by the Weizmann Institute of Science in Israel and XenneX Inc. of Cambridge Massachusetts, USA. (http://www.genecards.org) The webpage catalogues a large amount of sequence data including genomic, protein, recombinant protein, gene function, pathways and interactions, transcripts, primers, inhibitory sequences, and the like. Importantly there are links to commercial sources for biotechnology products related to the sequence information.

Untagged, full length human cDNA clones for BDNF, CNTF and NGF [Genes Hs.502182 (human brain), Hs.632114 (human white matter), and Hs.2561 (human retinoblastoma line) respectively] were purchased through ImaGenes GmbH, Berlin Germany. The sequences were housed in a pCMV-SPORT 6.1 vector (4.2 kb) (Figure 4.3).

**Figure 4.3:** Commercial vector pCMV-Sport6.1 housing the human neurotrophin sequences purchased through ImaGenes GmbH, Berlin Germany.
Limited sequencing (100-200 bp) was done by the company prior to sending cDNA to ensure it was the correct gene, and that the sequence was accurate across the span that was sequenced. The sequence for human NT3 was already available to the lab through our previous work developing lentiviral vectors. I had already cloned the human NT3 sequence into a lentiviral vector, pH-RCMV-GFP W Sin-18, 3 years prior to this project's onset. The gene material for GDNF was initially bought through ImaGene as well; however this came as a genomic clone housed in a pBeloBAC (bacterial artificial chromosome) vector. Given the presence of intron and exon DNA, the gene could not be amplified off the source material by PCR. The human GDNF sequence (variant 1) was subsequently obtained as a kind gift again from Dr. Thomas Ritter, supplied as a pBluescript vector.

4.2.2 General Methods of Molecular Cloning

Verification of commercial neurotrophin sequences by restriction digestion

Bacterial cell suspensions containing the neurotrophin plasmids were shipped in soft agar and were streaked onto Luria-Bertani (LB) agar plates containing 100 mg/ml ampicillin (LB-Amp plates) for selection of single colonies. Individual colonies were then selected with a sterile pipette tip and inoculated into 3 ml aliquots of LB broth with ampicillin, and again grown overnight with flask rotation at 37°C. 1.5 ml of the cell suspension was then placed onto Qiagen QIAprep MiniPrep™ columns for extraction and purification of the plasmid DNA, as per the manufacturer's protocol (Qiagen, Crawley, England). Briefly, the protocol relies on established methods of alkaline cell lysis and DNA denaturation, whereby bacterial chromosomal DNA precipitates upon correction of the pH and the plasmid DNA will correctly reform in solution. The Qiagen columns then rely on ionic conditions to bind plasmid DNA to column, allowing for serial washings, followed by elution of the DNA in pH neutral solute. Alternately, plasmid DNA was precipitated from the filtered, neutralized solute using 3 M sodium acetate with 1 volume of 100 % ethanol, pelleted rinsed in 70% ethanol and dried prior to re-dissolving in 25 mM Tris-Cl 10 mM EDTA pH 8.0. Purified DNA was stored at -20°C.

The purity and integrity of plasmid DNA was then assessed by restriction enzyme digests. Restriction digest reactions were carried out in volumes of 10 ul,
consisting of 1 ul DNA, 0.2-0.3 ul restriction enzyme and 1 ul 10x buffer, bringing the volume to 10 ul with nuclease-free water. Reactions were incubated overnight at 37°C, unless a quick screening was being used, and the reactions could be incubated for 3-4 hours. All restriction enzymes were obtained with their appropriate activity buffer from New England Biosciences (Bray, Ireland) as part of the company’s freezer program at REMEDI. Whenever possible, a ‘master-mix’ of enzyme, buffer and water was prepared for the required number of reactions plus two, saving enzyme use. When 2 different enzymes were required, buffer compatibility was determined as per the NEB cross-table, adding bovine serum albumen to 1x from 10x stocks as recommended.

To assess the ‘health’ of the purchased plasmids, uncut and cut plasmid vector were run on 1% at agarose gels in 25 mM tris acetate-EDTA (TAE), in TAE electrophoresis buffer at 80-120 V. DNA banding was visualized with SYBR-Safe™ dye (Invitrogen) instead of ethidium bromide, which was instilled directly into the molten agar mixture prior to pouring the gel. DNA fragments were sized in comparison to a standard 1 kb ladder (NEB). Minigels of 80 ml agar-TAE solution typically were used, with 10 space combs allowing for the loading of 10 ul of DNA plus loading buffer. The initial screen involved cutting out the gene insert, as well as demonstrating the expected presence of an internal restriction site based upon the known neurotrophin sequences. Gene sequences were submitted to New England Biosciences ‘NEB Cutter’ website (http://tools.neb.com/NEBcutter2) to identify restriction sites, particularly single cutters within the gene that were offset to one end, which would then indicate clone directionality when paired with a site external to the gene.

For pCMV-SPORT6-NGF, the total plasmid size was calculated to be about 5.7 kb including the gene insert of 726 base pairs. The template plasmid was demonstrated to correctly cut with XbaI-EcoRV to remove the insert gene, as well as with EcoRI to remove a skewed length of the gene via an internal and external site pair. For pCMV-SPORT6-CNTF, the construct was 6.2 kb including an insert size of 2100 bp, 603 bp of which made up the gene of interest. This plasmid was shown to cut correctly with EcoRI and NotI to remove the insert, and with HindIII to remove a portion of the gene via an internal and external site pair. For pCMV-SPORT6-BDNF, no internal sites of use could be
identified and the gene was amplified directly off the source vector without restriction analysis.

**Plasmid amplification and storage**

Once a particular bacterial colony was verified to contain the correct plasmid upon restriction digest analysis, the remaining 1.5 mls of its miniprep culture were inoculated into 500 mls of LB broth with ampicillin and grown overnight at 37°C. The plasmid DNA was then extracted again using QIAfilter™ Plasmid Maxi Kit from Qiagen, a larger scale purification based on the same principles as the Miniprep. A portion of the cell suspension was stored at -80 as a glycerol stock (15% glycerol). Where plasmid came as a lyophilized source, or where the purified plasmid needed to be amplified, 1 ul of the plasmid DNA stocks was transformed into competent E.Coli cells, One Shot MAX-Efficiency DH5a-T1 (Invitrogen), according to manufacturer's protocol. Briefly the competent cells were supplied in 50 ul aliquots at -80°C, which were then thawed on ice. Upon introduction of the DNA, the cells were mixed by flicking the tube gently and incubating on ice for 30 minutes. The vials were then placed in a 42°C water bath for 30 seconds to heat shock them, which improves transformation efficiency. 250 µL of warmed SOC media (as supplied) were then added to the cells, following which they were incubated with rotation of the vials for one hour at 37°C and at 225 rpm. The cell suspension was then plated on LB-agar plates with the appropriate antibiotic, most commonly ampicillin 0.1 mg/ml, and allowed to grow into colonies overnight.

**PCR amplification**

A PCR based strategy was used to amplify neurotrophin sequences from the source plasmids. The rational for using PCR was to allow for creation of restriction sites required for insertion of the neurotrophin into the multiple cloning site of pLXSN-IRES-eGFP retroviral expression vector. PCR primers for the 5’ end were designed to first incorporate the needed restriction site sequence, then include the Kozak sequence (GCCACC) followed by the first 5’→3’ 18 base pairs of the neurotrophin. The Kozak sequence (Kozak, 1990) is a ribosomal targeting sequence for optimal translation efficiency. The 3’ PCR primer was designed to be the needed restriction site sequence followed by the reverse complement of the
last 18 base pairs of the neurotrophin. Please see Appendix 1 for details of the human neurotrophin cDNA sequences, translated protein sequences and primers used for an amplification off of the target source. All primers were custom synthesized and supplied a lyophilized oligo-DNA salts by Eurofins MWG/Operon, Ebersberg, Germany.

Safeguards were taken to minimize introduction of copy errors during template amplification. To ensure accuracy, a ‘GC rich’ PCR kit from Roche Applied Science (Burgess Hill, England) was used. The PCR reaction was carried out as a 25 µL mixture, using 50 picomole of forward and reverse primers with 25 mM of each NTP, and 0.5 µL of template DNA. An ultra high fidelity DNA (pfu) polymerase was used in combination with Taq polymerase to maximize copy accuracy, 1.25 units of enzyme, as well as to create TA overhangs necessary for further cloning steps. The reaction mix was as follows:

5x buffer - 10 ul
Resolution solution - 5 ul
mg 2+ salt solution - 3 ul
forward primer - 0.5 ul (100pmol/ul stock)
reverse primer - 0.5 ul
dNTP (from stock 100 mM total, 25 mM each NTP) - 0.4 ul
PCR water - 28.6 ul
Enzyme mix 1 ul.

The kit contained a proprietary "resolution solution" designed to more cleanly amplify difficult templates rich in guanine and cytosine content, as well as magnesium solution and PCR grade water. The PCR cycling was set for 95°C for 2 minutes initial melting, 30 cycles of 95°C for 30 seconds melting, 52°C for 30 seconds annealing, 72°C for one minute extension, followed by 72°C for 10 minutes final extension and holding at 4°C. The annealing temp was set as being 5°C less than the lower of the two primer's melting temperature (Tm).

**Topo-Isomerase cloning of the PCR product**

The PCR mixture was then run on a 1% agarose gel with wells deep enough to hold the entire reaction mix. The PCR product band size was verified as
being correct for expected amplicon, and the fragment was excised with a metal scalpel blade. The DNA was gel purified using a Wizard SV™ Gel and PCR Clean-Up system (Promega, Southampton, England). The protocol utilizes membrane binding of the DNA with serial washes of detergent and ethanol, followed by the elution of the DNA in 20 µL nuclease-free water.

Following the purification of the amplified target, the product was cloned into an intermediary vector, using a Thymidine-Adenosine (TA) cloning kit (Invitrogen). TA cloning utilizes a topo-isomerase enzyme covalently linked onto a shuttle vector (pCRII-TOPO vector (3.9 kb)) to bind TA overhangs on the PCR product and ligate the fragment into the vector. TA overhangs are created on the DNA template during PCR amplification provided the mix contains Taq polymerase. The pTOPO vector has a multiple cloning site allowing for further flexibility of product excision-ligation, as well as sequencing primer sites, the M13 reverse and forward sites and the Sp6 and T7 sites.

The TA cloning reaction was highly efficient. The reaction occurs within a 5 minute incubation time on the bench at room temperature and the reaction mix is immediately transformed into competent cells for colony selection. It is a non-directional ligation yielding roughly 50% of clones forward and backward; restriction enzymes used to remove the ligand again provide for clone sequence directionality. A further advantage of using the intermediate vector was to provide the physical structure of surrounding DNA in order to cleanly cut the neurotrophin free with the necessary restriction enzymes. Enzymes cut at their restriction site more efficiently with surrounding shoulder sequences than if the purified PCR fragments were cut on their own. A reaction mix of 2 µl of purified gel product, 0.5 of salt solution and 0.5 of the TOPO vector was used. The entire reaction mix was transformed into DH5α E.Coli as described. Minipreps were from the colony plate and the purified TOPO vectors were cut with EcoRI to screen for the insert. The TOPO vector has two EcoRI sites flanking the PCR product insertion site, allowing for easy screening of product insertion. Positive cultures for each neurotrophin were grown as maxi-preps and stored as glycerol stocks for banking as TOPO clones for future use.
4.2.3 Generation of a pLXSN-IRES-eGFP Neurotrophin Library

Following amplification of the target human neurotrophin sequence, and insertion into the pTOPO vector, the inserts were sequenced. Sequencing was done at the Mayo Clinic, Rochester MN in the DNA Sequencing Core, Stabile Building. To prepare the samples 1.6 picomole of sequencing primer (M13 forward and reverse) was combined with 0.25 ug of template TOPO plasmid, with 0.5 µL DMSO for GC rich samples, bringing the volume to 6 µL with sterile water in 0.2 ml MicroAmp strip tubes. Sequence accuracy was determined by BLAST alignment with the NCBI Reference sequences. With confirmation of sequence accuracy, the neurotrophic sequences were excised from the TOPO vector using the restriction sites built into their original primer design, digesting with excess enzymes overnight to ensure complete excision of the fragment. The pLXSN-IRES-eGFP vector was also digested overnight with the same enzymes as the TOPO fragment. The linearized vector backbone was treated with Antarctic Alkaline Phosphatase (NEB) to remove 5' phosphate groups and limit self re-ligation. Five units of phosphatase enzyme were added to 1 ug of vector DNA, incubated for 15 minutes at 37°C, and heat inactivated five minutes at 65°C prior to use.

For NT-3 and CNTF, primers containing EcoRI and SalI were used for the 5’ and 3’ sites respectively. For NGF, EcoRI could not be used as there was an internal EcoRI site; the plasmid was then cloned by single digest with SalI, which did add complication in terms of verifying the gene orientation within the clone. For BDNF, MluI forward primer and SalI reverse primer sites were used for amplification, but the TOPO clone was cut with EcoRI and SalI for insertion. GDNF was amplified and excised using Mlu forward and SalI given an internal EcoRI site.

For sequence ligation, linearized and de-phosphorylated pLXSN-IRES-eGFP vector and excised neurotrophin fragment insert were run on a 1% gel and gel purified as described above. The concentration of DNA in ng/ul was measured using a NanoDrop spectrophotometer (Thermo Scientific, Willington Delaware). This value was converted into pmol of ends per ul using the Biomath calculator available on the Promega website (http://www.promega.com/en-us/resources/tools/biomath-calculators). The conversion is based on the length of
the DNA fragment and on the average molecular weight of a single nucleotide pair. Having calculated picomole of ends, the ligation reaction was set up to have a 3:1 insert:vector ratio. 10 units of DNA ligase (Sigma) were combined with vector and insert volumes with 5X reaction buffer (Invitrogen) containing ATP and MgCl2, in a total reaction volume of 10 ul. The reaction was incubated overnight at room temperature. In the morning half of the reaction was then transformed into DH5α cells, plating 150 ul of the transformant cell suspension and selecting all available colonies for screening of the correct construct. Constructs were digested with restriction enzymes corresponding to their ligation sites, such that they could first be screened for insert. Subsequently screening for correct 5'→3' insert orientation was done using corresponding sites within and outside of the insert. Following identification of the correct pLXSN-IRES-eGFP neurotrophin construct, the gene inserts were again sequenced, and compared via BLAST alignments to the source sequence as well as the preliminary TOPO clone sequences.

4.2.4 Confirmation of Bistronic Neurotrophin and eGFP Expression

To confirm the expression of the neurotrophic protein, 293T cells were transfected in triplicate with pLXSN-IRES-eGFP NT3, NGF, GDNF and BDNF constructs. 0.8 ug of DNA were transfected onto cells seeded at 25,000 cells per well in a 24-well plate in triplicate. The plasmid was introduced in antibiotic free media using Lipofectamine 2000 as the transfection reagent (Invitrogen) according to the manufacturer's protocol. 8 µL of Lipofectamine were added to 192 ul of Optimem media, 50 uL of which was mixed with 50 µL of DNA/Optimem solution and placed over the cell monolayer. The cells were exposed to the plasmid and reagent for 24 hours following which full cell culture media with antibiotic was replaced. The media was assayed by enzyme linked immunosorbant assay (ELISA) after 72 hours. ELISA kits for human NT3, NGF, BDNF and GDNF were purchased from R&D Systems Europe (Abingdon, England). Media from non-transfected 293T cells was used as negative control.

Each kit contains lyophilized neurotrophin protein for dilution and creation of a standard curve. For NT3, stock protein at a concentration of 50 ng/ml was diluted in 0.5 ml 1% BSA. From this stock aliquots of 2, 4, 8, 12, 16, 20, and
30 μl were taken, made up to a volume of 1000 μl with 1% BSA. For NGF, stock protein of 80 ng/ml was diluted in 0.5 ml 1% BSA with aliquots of 0.625, 1.25, 2.5, 5, 7.5, 10, 12.5 and 18.75 μl brought up to a volume of 1 ml. For BDNF, 170 ng/ml stock was diluted in 0.5 ml 1% BSA with aliquots of 0.29, 0.58, 1.17, 2.35, 3.53, 4.7, 5.88, 8.82 brought up to a volume of 1000 μl. These dilutions yielded a standard curve of 100, 200, 400, 600, 800, 1000 and 1500 pg/ml for each neurotrophin. Capture antibodies were diluted in PBS to working concentrations of 0.5 μg/ml for NT3, 2.0 μg/ml for NGF, 2.0 μg/ml for GDNF, and 2.0 μg/ml BDNF. Detection antibodies were diluted in 1% BSA to working concentrations of 0.2 μg/ml for NT3, 0.05 μg/ml for NGF, 0.1 μg/ml for GDNF, and 0.025 μg/ml for BDNF. The assay procedure was carried out according to the manufacturer’s protocol. The wells of 96 well flat bottomed ELISA plates were coated overnight with capture antibody and washed three times with wash buffer (0.05% Tween 20 in PBS pH7.2-7.4). 100 μl of cell culture media containing the secreted neurotrophic factor was added to wells in triplicate, incubated for two hours and washed three times. Detection antibody was then added to the well, incubated for two hours and washed three times, at which point 100 μL of diluted Streptavidin-Horseradish Peroxidase (HRP) was incubated with the sample for 20 minutes. Following a final three washes, the substrate solution (a 1:1 mixture of Colour Reagent A (H2O2) and Colour Reagent B (tetramethylbenzidine)) was allowed to develop in the dark prior to the addition of 2 N H2SO4 to stop the reaction. The sample absorbance was then read using a microplate reader set to 450 nm with wavelength correction at 540 nm.

Using the same cells as were transfected for neurotrophin ELISA, eGFP expression off of the IRES motif was assessed by GUAVA flow cytometry (Millipore, Billerica, Massachusetts, USA). Cells within the 24 well plate were washed with PBS and trypsinized using 200 μl 0.25% trypsin for 10 minutes, following which the well was rinsed and the reaction stopped with 300 μl cell media. The suspension was then fixed with 400 μl 4% paraformaldehyde in PBS for 20 minutes or more, redispersed and aliquoted into epindorf tubes. The cells were pelleted at 400 g for 5 minutes and the supernatant poured away, resuspending the pellet in 400 μl PBS. 200 μl of the preparation were loaded into round-bottomed wells of a 96 well plate, and read by the GUAVA machine after appropriate gating. Results are reported as the percentage of GFP positive cells.
4.2.5 Retroviral Production using GP+E86 Packaging Cell Lines.

Upon confirmation of bistronic expression of the neurotrophic factors along with eGFP, we could proceed with the establishment of retroviral producing packaging cell lines. GP+E86 cells were obtained as a gift from the Dr. Ritter's lab. The retroviral expression vector contains the viral long terminal repeats (LTR) and the $\psi^+$ packaging sequence. The LTR contains promotor elements initiating the transcription of the viral RNA but lacks retroviral $gag$, $pol$, and $env$ gene sequences necessary for particle formation and genome replication. Packaging cell lines are designed to synthesize retroviral proteins required for assembly of high titre virus in a manner that removes the need to use helper viruses supplying such elements in trans. The danger of using helper virus constructs lies in the possibility that a replication-competent virus could be generated through recombination events, in which the intact $\psi^+$ packaging sequence of the expression vector corrects the deleted $\psi^+$ sequence of the helper virus. Wild type virus risks proliferation and multiple genomic integration with oncogene activation (Miller, 1990).

The GP+E86 is a packaging cell line designed at Columbia University in the late 1980s by Dina Markowitz and colleagues (Markowitz et al., 1990). The group created a safe and efficient NIH-3T3 cell line by electroporation, separating the viral $gag$, $pol$ and $env$ genes of the helper virus on two separate plasmid sources. As the name infers, $gag$ and $pol$ are on one plasmid, $env$ is on another; E86 refers to an ectropic envelope protein, which limits viral infection rat or murine cells, further adding safety to the vector. The $\psi^+$ packaging sequence along with the 3’ LTR are deleted from both plasmids, limiting the possibility that the viral genes ($gag$, $pol$ $env$) will be encapsulated into the virion to produce a replication-competent virus. Once the packaging line is transfected with a retroviral expression vector that contains a packaging signal, the viral transcript of the expression vector, containing the gene inserts and selectable antibiotic marker, are packaged into infectious vector particles within 48-72 hours.

On Day 1, GP+E86 cells were trypsinized and sub-confluently seeded at a concentration of 80,000 cells per ml on 10 cm dishes. The cells were easy to maintain and are grown in DMEM with 10 % fetal calf serum with antibiotic. The
following day, 25 µg of retroviral expression vector, pLXSN-IRES-eGFP BDNF, NT3 or GDNF, was introduced into the cell line via transfection. The transfection reagent jetPEI™ (PolyPlus Transfection, Illkirch, France) is a linear polyethylenimine (PEI) derivative for cationic gene delivery. GP+E86 cells were transfected according to the manufacturers protocol, whereby DNA was added up to a volume of 2 ml in sterile 150 mM NaCl as supplied in the kit. In a second vial, 220 µl of the PEI reagent were added to 150 mM NaCl to a volume of 2 ml. The reagent solution volume was added slowly to the DNA solution and incubated for 30 minutes in order to form PEI-plasmid transfection complexes. The 4 ml combined volume containing complexes was introduced in a dropwise fashion to the cell culture with gentle mixing. After 24 hours (Day 3), the media was changed to warmed growth media.

On Day 4, a second batch of GP+E86 cells were plated at the same density on 10 cm dishes. These cells were pre-treated for 16 hours with 100 ng/ml tunicamycin (in 70 mM NaOH). By the following day (Day 5), cell media from the previously transfected line now contained a low titre retrovirus, which was then used to replace the media on the second batch of tunicamycin pre-treated packaging cells. The idea was to now use low titre virus to transfect the packaging line for the stable and perhaps multi-site integration of the expression vector sequences, allowing for more efficient viral production from the cell line. Pretreated GP+E86 cells were incubated with low titre viral supernatant in the presence of 8 µg/ml of polybrene (1.5-dimethyl-1.5-dizaundeca-methylene polymethobromide (Sigma, stock 1 mg/ml in PBS)). Polybrene improves viral cell receptor binding and particle uptake. 24 hours following the transfer of viral media onto the packaging line, the media was changed to growth media containing 1 mg/ml G418 (neomycin analogue) for the selection of positively transduced cells. Over the course of the next 10 days, cells were intermittently monitored for eGFP expression, and cell debris of non-liable cells was rinsed clear every 2 days with fresh media containing the selection antibiotic. Instead of picking individual colonies of cells, the entire plate was trypsized after 10 days of selection, and reseeded for further growth and stock storage as a mature packaging line. In total, 2 batches of GP+E86 packaging lines were made and analyzed for each of the NT3, GDNF, and BDNF retroviral constructs.
Working safely with retrovirus

A series of 12 separate standard operating procedure (SOP) documents were prepared to bring both the Mayo Clinic lab and the virus room at NUI Galway in compliance with government and institutional regulations regarding working safely with genetically modified organisms (GMO). These documents related to cleaning and disinfection of equipment, transport within the facility, waste disposal, training of new personnel, and the overall safety of day-to-day work with the viral vectors. Work with retroviral vectors was done in a dedicated room with specifically assigned tissue culture hoods, certified class II biological safety cabinets with vacuum lines equipped with an in-line HEPA filter as well as a primary and secondary vacuum flask containing 10% bleach solution on a freshly prepared 1% Virkon solution. SOPs were present at all times in the room for reference. All materials that came into contact with the retrovirus media namely pipette tips and tissue culture flasks, were disinfected using 1% Virkon solution for 30 minutes, prior to be autoclaved for disposal. Liquid waste was also mixed with Virkon or bleach prior to being autoclaved for disposal. All work surfaces were disinfected with 1% Virkon followed by 85% ethanol solution before and following use.

Estimation of retroviral titre

A serial dilution method was employed to estimate the titre of the retroviral vector particles in colony-forming units (CFU). NIH 3T3 cells were seeded at 7.5 x 10^4 cells per well of a 6 well plate. Retroviral supernatant media from the packaging line was placed onto the cells in increasing 10-fold dilutions, from neat retroviral media to a dilution of 1:100,000 in the presence of 8 ug/ml polybrene and incubated for 24 hours. The media was replaced after 24 hours with fresh DMEM with 10% FBS growth media with 1 mg/ml G418 selection antibiotic, changing again every 2 days. After a total of 12 days in culture, the number of cell colonies present in each well was counted. The titre was calculated as being the number of colonies (n) present at the dilution (10^x) which preceded the first dilution in which no colonies were seen, and was represented as n x 10^x colony forming units, or ‘G418-resistant units’ (GRU).

A second method involved plating NIH-3T3 cells at the density of 2 x 10^4 cells per well in a 12 well plate. The viral supernatant media was then diluted in a
series of six four-fold dilutions. For comparison, viral media that had been produced from the initial plasmid transfection (low titre virus) was analyzed in parallel with viral media obtained from the final packaging line preparation. Following incubation of the cells for 24 hours under viral media, the media was changed to DMEM + 10% FBS for an additional 48 hours. At that time the cells were washed with PBS and trypsinized using 200 ul 0.25% trypsin for 10 minutes, following which the well was rinsed with 300 ul cell media. The suspension was fixed with 400 ul 4% paraformaldehyde in PBS for 20 minutes or more, redispersed and aliquoted into Eppendorf tubes. The cells were pelleted at 400 g for 5 minutes and the supernatant poured away, resuspending the pellet in 400 ul PBS. 200 ul of the preparation were loaded into round-bottomed wells of a 96 well plate, and read by the GUAVA machine after appropriate gating. Viral titre was calculated in transfection units (TU) as the percentage of GFP positive cells/100 multiplied by number of cells plated (Cashion et al., 1999) per unit virus volume in the well. Titres are most accurate when calculated in to 1 – 20 % GFP positive range.

To amplify the viral titres, supernatant media from the packaging line was concentrated using Vivaspin 20 ml ultrafiltration spin columns (Sartorius Stedim Biotech GmbH, Goettingen, Germany) with Molecular Weight Cut Off (MWCO) size < 100-300,000 PES, for 30 minutes at 3,000 x g.

Retroviral delivery of the eGFP-neurotrophin construct to NIH-3T3 cells

NIH-3T3 cells were used measuring the expression of eGFP and the neurotrophic factor following infection with retrovirus. This cell line is considered standard for retroviral assays given ease with which it is infected. NIH-3T3 cells were plated at the density of 2 x 10^4 cells per well in a 12 well plate in triplicate. The viral supernatant media was then diluted in a series of six four-fold dilutions. Following incubation of the cells for 24 hours under viral media, the media was changed to DMEM + 10% FBS for an additional 48 hours. Cell media was removed and further diluted in a ratio of 1:50, 100 ul of which was assayed by ELISA to detect the neurotrophin protein (pg/ml). Media from NIH-3T3 cells not infected with retroviral media but subjected to the same culture conditions was used as the negative control.
4.2.5 Neurotrophic Schwann Cell and Mesenchymal Stem Cell Lines

With confirmation of a functional, high titre retrovirus delivering the neurotrophic factor with eGFP, our attention turned to producing stably transfected Schwann cell and MSC lines. Schwann cells were derived from neonatal rat sciatic nerve and used within 72 hours as a primary culture for scaffold loading as detailed in chapter 2. Several modifications to the cell culture protocol were made to allow the cells to grow in culture for the time required for antibiotic selection of virally transduced cells. Upon antibiotic selection, the cells needed to be robust enough to be passaged several times in order to obtain the required cell numbers for scaffold loading. These modifications were based on a literature review for the isolation and purification of primary Schwann cells. On that basis the culture media was modified to contain 10 ng per ml of the growth factor neuregulin [glial growth factor 2], (R&D Systems, Minneapolis MN USA), along with 2 uM forskolin (Evans et al., 1985) (Sigma).

Primary Schwann cells were seeded at a density of 80,000 cells per ml in 25 cm² flasks. After 24 hours cell culture media was replaced with viral supernatant from NT-3, GDNF or BDNF retroviral packaging line cells in the presence of polybrene. Viral supernatant media was not filtered given that the virus binds to the filter material. Media was centrifuged however to pellet any viable packaging cell which may have been floating in the supernatant. This reduced the possibility that the target cell line would be contaminated by the packaging cell type, in which case colonies of course would form and grow in abundance in the presence of the antibiotic selection.

Cultures were also carefully assessed for their morphology, the spindle shaped Schwann cell looking characteristically different to the fibroblast GP + E86 cell. The target cells were infected for 24 hours, replacing the media with Schwann cell media containing neuregulin, forskolin and G418 selection antibiotic. The cells were maintained for at 10-12 days, changing the media every two days to feed the cells and to remove cell debris. Small colonies of transduced cells became apparent after 3-4 days, and were allowed to grow to confluence, prior to trypsinizing and expanding the culture in modified media without antibiotic selection. The cell lines were assessed using fluorescent microscopy for...
the presence of eGFP and stained with S100 antibody using a Cy-3 labeled secondary antibody. Cells were harvested and stored as stocks in liquid nitrogen. MSCs at low passage were similarly plated at 80,000 cells per ml in 25 cm² flasks, and in the same manner as Schwann cells, were cultured in the presence of MSC growth media with G418 antibiotic selection following incubation with viral supernatant media taken from the NT-3, GDNF, or BDNF packaging cell line. Media was changed every two days to feed the cells and to remove cellular debris, and after 10-12 days, the colonies were grown in normal MSC growth media to confluency.

**Dorsal root ganglia isolation and neurite outgrowth**

Whole dorsal root ganglia DRG were isolated from embryonic rat pups and plated onto collagen-coated dishes. To obtain the DRGs, a pregnant female Sprague Dawley rat at embryonic day 15 was anaesthetized with 100 mg/kg of pentobarbital and then euthanized with intracardiac injection of an additional 25 mg. Under sterile surgical conditions, the abdomen of the animal was opened, the uterus removed, the embryonic pups were dissected clear of the placenta and placed into 100 mm dishes containing L-15 culture medium (Gibco BRL). The pups were placed under a dissecting scope. Incisions were made in parallel using micro-scissors lateral to the spinal column which was removed en masse. The column was placed on its dorsal side and incisions were made along both sides to remove the vertebral bodies. The exposed spinal cords were removed from the vertebral arches and placed in a 60 mm dish containing L-15 media. Individual DRG were pulled free from the cord with forceps, rinsed in media, and plated with one drop of media in groups of four on 35 mm tissue culture dishes coated with collagen. The dishes were incubated at 37°C for one hour prior to the addition of another 20 drops of media as needed to cover the DRG. Care was taken not to dislodge the culture from the base of the dish.

To test the functionality of NT-3 produced and secreted by the genetically modified Schwann cell line, freshly plated DRGs were submerged in cell culture media taken directly off of a confluent culture of stably transduced Schwann cells derived from infection with eGFP + NT-3 retrovirus. The media was diluted 1:10, 1:2, and used neat. As a control, Schwann cell growth media including neuregulin and forskolin, was used. The DRGs were visualized using light microscopy after
48 hours of incubation and assess for the degree of new neurite outgrowth in response to transduced Schwann cell supernatant media.

4.3 RESULTS

4.3.1 Neurotrophic pLXSN-IRES-eGFP Plasmid Constructs

cDNA genetic sequences for five human neurotrophic genes, neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glia-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CTNF) were purchased or obtained from existing lab stocks. Using a PCR based cloning strategy, each neurotrophic sequence was amplified off of the plasmid source using primers that introduced unique restriction enzyme sites to the 5’ and 3’ ends, and added the Kozak ribosomal targeting sequence \([\text{ACCG CC}]\) to the 5’ end. Care was taken in the PCR protocol to use a high fidelity polymerase and chemical modification for difficult GC rich sequences. Each amplicon was subcloned into a pTOPO vector for sequencing and banking, prior to being ligated into the retroviral expression plasmid pLXSN-IRES-eGFP. The plasmid contains the long terminal repeat sequences encoding enhancer and promoter elements derived from of the Moloney Murine sarcoma virus, along with the \(\psi^+\) packaging sequence and a neomycin resistance gene. The presence of an internal ribosomal entry site (IRES) allows for translation of the neurotrophic factor and eGFP from a single by bistronic mRNA.

The plasmid constructs were verified on two levels, first by restriction digests to check for insert orientation and gross sequence size, and secondly by sequencing at three different time points in their construction. Limited initial sequencing was done by the supplying company. The intermediate TOPO were sequenced using M13 primer sites from a 5’ sense and 3’ antisense direction, covering the length of the gene. The final constructs were also sequenced from both directions. In each case, the sequences were aligned with the NCBI reference sequence using NCBI BLAST alignments to verify accuracy along the entire length. Only one sequence error was noted. This occurred as a point mutation in the 3’ end of both the NGF TOPO clone and in the pLXSN-bNGF-IRES-eGFP...
final construct. This change resulted in a lysine to proline substitution, which unfortunately had implications for protein folding. At this point, the NGF clones were discarded, and the decision was made to proceed with constructs for NT-3, BDNF, and GDNF.

A vector map for a representative construct is detailed in Figure 4.4.

**Figure 4.4:** pLXSN-IRES-eGFP retroviral expression vector encodes the cDNA genes for human NT3, GNDF, CNTF, and bNGF as a single bistronic mRNA transcript with eGFP, given the presence of an internal ribosomal entry site (IRES). GDNF is shown as representative of the group. Please see Appendix 1 for details of the sequences. Expression of the neurotrophin with eGFP is driven by promotor elements within the Moloney murine sarcoma virus LTR. The ψ+ packaging sequence orchestrates viral particle assembly when gag, pol and env are supplied in trans by the GP + E86 packaging cell line. The gene for neomycin resistance is under the control of a sarcovirus (SV40) promotor and, allows for selection of cells transduced by plasmid or virus in the presence of the antibiotic analogue G418. pLXSN nomenclature refers to the 5’ to 3’ orientation of retroviral elements, the LTR (‘L’), the ψ+ sequence (‘X’), the SV40 promotor (‘S’) driving Neomycin (‘N’) resistance genes.

**Bistronic neurotrophin and eGFP expression**

To confirm that the plasmid constructs were expressing their respective neurotrophin and eGFP, 293T cells were transfect with with pLXSN-IRES-eGFP.
BDNF, NT3 and GDNF and NGF plasmid. The transfection was done over 24 hours using Lipofectamine 2000, and the cells were assayed after an additional 48 hours in culture. The concentration of neurotrophin protein eluted into the supernatant media was measured by ELISA, and the percentage of cells expressing eGFP was measured by GUAVA flow cytometry. As seen in Figure 4.5, the total concentration of BDNF was 1.051 +/- 0.011 ng/ml of supernatant media, for NT3 was 1.478 +/- 0.008 ng/ml and for GDNF was 0.619 +/- 0.117 ng/ml. Notably, the transfection efficiency using Lipofectamine was low, as assessed by % GFP positivity, whereby 30.27 +/- 0.58 %, 41.51 +/- 1.12 % and 41.83 +/- 1.5 % of cells expressed eGFP when transfected with the BDNF, NT3 and NGF plasmids respectively. To some degree variable efficiency could account for the discrepancy in neurotrophin concentrations between the plasmids, particularly as the genes were driven by the same promoter elements.

![Graph showing total pg/ml of human BDNF, NT3 and GDNF protein levels following transfection of the pLXSN-IRES-eGFP plasmids into 293T cells. Transfection efficiency is listed as % of eGFP positive cells after 72 hours in culture.]

**Figure 4.5:** Total concentration (pg/ml) of human BDNF, NT3 and GDNF protein levels following transfection of the pLXSN-IRES-eGFP plasmids into 293T cells. Transfection efficiency is listed as % of eGFP positive cells after 72 hours in culture.
No NGF protein was detectible in the supernatant media following pLXSN-NGF-IRES-eGFP plasmid transfection. DNA sequencing of the NGF construct had shown a point mutation in the encoding sequence that resulted in the substitution of lysine to proline residue. Such a change would significantly affect protein folding in that the proline is a larger and more hydrophobic amino acid. NGF was detectible on ELISA following transfection of 3 different clones of the original pCMV-SPORT6 plasmid, indicating the sequence mutation was likely a consequence of the PCR-based cloning process (Figure 4.6). The level of expression from the CMV promotor vector was similar to that seen by LTR promotor elements in the other plasmids.

![Final NGF Conc. (pg/ml)]

**Figure 4.6:** 3 separate clones of the source NGF plasmid (pCMV-SPORT6) produce around 920 pg/ml of NGF protein, but no NGF protein is detectible from the pLXSN plasmid following PCR amplification and cloning from the source.

### 4.3.2 Retroviral Packaging Cell Lines

With confirmation of neurotrophin and eGFP expression from the retroviral vector, GP + E86 packaging lines were made to produce infectious vector particles for gene delivery. The GP + E86 is an NIH 3T3 based cell line stably transfected with separate plasmids supplying retroviral *gag* and *pol*, and the ecotropic envelope protein (E86) *in trans*. In the presence of the LTR and $\psi^+$ sequences from the expression vector, infectious viral particles are efficiently packaged with the genes of therapeutic interest, and without genetic elements of the GP + E86 genes. The separation of plasmids, among other sequence...
modifications to the $\psi^+$ and LTR sequences, safeguards against the vector from becoming replication competent.

GP + E86 packaging lines were created in 2 batches for each of the three neurotrophic vectors, NT3, GDNF and BDNF. The cell lines were made in a two-step process. The packaging line was initially transfected with retroviral expression vector plasmid to create a low titre virus. This virus was then used to infect a second batch of packaging cells, with the potential for higher efficiencies of viral production with multiple integrations of the expression vector sequence. One day following viral infection of the cell line, cells were selected in the presence of 1 mg/ml G418 neomycin analogue, allowing the formation of colonies of successfully transduced cells. In Figure 4.7, eGFP fluorescence within the GP + E86 monolayer can be seen to be patchy and of low density following retroviral expression plasmid transfection. At this stage the transfection efficiency was poor at least on visualization, but sufficient to produce a low titre virus for re-infection of a fresh GP + E86 line.

Figure 4.7 Cells transfected with the pLXSN-IRES-eGFP neurotrophin plasmids express eGFP sporadically, suggesting a low efficiency of plamid transfection. This efficiency was sufficient to produce a low titre retrovirus for re-infection of the packaging line to improve viral production. Magnification 20x.
Following several days of cell selection with G418, washing the cultures free of dead cell debris and replenishing the line with nutrients and fresh antibiotic, colonies were seen to expand from small groupings of surviving cells. The typical appearance of a colony is seen in Figure 4.8 at 4x magnification. The same colony is seen under higher power (10x magnification) this time with eGFP fluorescence. The cells are often of variable eGFP intensity, likely a reflection of different levels of eGFP protein expression following multiple vector insertions into the genome of several cells. This phenomenon similarly can be seen at 20x power in a colony which has grown to confluence (C). After 12 days of selection, the plate was trypsinized to disperse the colonies and cell suspensions of the mature retroviral packaging lines were either grown to augment cell numbers or frozen at low passage.

**Figure 4.8:** GP + E86 cells infected by the retroviral expression vector delivering eGFP (with the neurotrophic factor) form colonies within days of cell selection with G418 neomycin analogue. The cells are seen to be of variable eGFP intensity suggesting there may have been variable numbers of integration events of the expression vector sequences.
**Retroviral titre estimation**

Two methods were used to determine titre of the retrovirus produced by the packaging line. In the first method, NIH 3T3 cells were infected with retroviral supernatant media in increasing dilutions of 4x for 24 hours prior to cell selection with growth media containing G418 for 12 days. By this method the number of surviving colonies at the highest viral dilution (immediately preceding the dilution supporting no colony growth) provides a value of colony forming units. Titres of NT3, BDNF and GDNF retrovirus were estimated to be $1.6 \times 10^6$, $1.7 \times 10^6$ and $2.0 \times 10^6$ colony forming units (cfu) respectively. We found that the titres could be significantly improved by concentrating the supernatant viral media with filter centrifugation. Serial dilutions of the concentrate showed the titres to have increased to of $2 \times 10^{10}$, $1 \times 10^8$, and $2 \times 10^{10}$ cfu respectively for NT3, BDNF and GDNF retrovirus.

![NIH 3T3 Retroviral Transduction](image)

**Figure 4.9:** NIH-3T3 cell transduction by viral supernatant derived from the mature retroviral packaging lines is compared to supernatant following plasmid transfection with the retroviral expression vector. Dose dependant responses in the target cells are seen.

In the second method, four fold serial dilutions of viral supernatant were again applied to NIH 3T3 cells for 24 hours, but without subsequent cell selection with antibiotic media. Instead the percentage of cells expressing eGFP after 72
hours in culture was measured by GUAVA flow cytometry. In this experiment we compared the percentage of cells at serial dilutions following retroviral expression plasmid transfection and retroviral supernatant media from the mature cell line. This gave an indication of the viral titre that was used to re-infect the GP + E86 line in the second phase of packaging line production, as well as the viral titre produced by the mature line as applicable to future cell line production. In Figure 4.9 the percentage of GFP positive cells is plotted against the log of the viral dilution factor for each viral or plasmid condition, demonstrating a dose dependant response in the target cells, and distinguishing the low titre virus from the mature packaging line virus. Viral titre of mature packaging cell supernatant was calculated to be between 3 and 6 x 10^6 transducing units. The low titre virus initially produced from plasmid transfection was on the order of 5 x 10^4 transducing units.

No data points were available for neat, viral supernatant, from the mature packaging line, as the application of undiluted viral supernatant killed off the recipient 3T3 cells. Cell death indicates lethality or apoptosis induced by multiple genomic integrations of the retroviral sequences. Between 85 to 95 % of the cells were transduced at a 4 fold dilution of media. For the low titre virus, undiluted media could only transduce between 40 and 60% of the target 3T3 cells, indicating that using virus in the second phase of packaging line production improved the efficiency of the line (in addition to antibiotic cell selection).

**Neurotrophin expression following retroviral gene delivery**

To assess the production of neurotrophin protein following retroviral infection, NIH 3T3 cells grown in the presence of 4 fold dilutions of viral supernatant media for 24 hours. Media was taken from the same cells as were used to measure eGFP dose response and titre calculation. The concentration of neurotrophin eluted from the genetically modified cells was assessed after 72 hours growth in culture. Whereas neat supernatant killed the target cell, expression of the neurotrophic factor was detectible at the first dilution step and subsequently found to be dose-dependent based on the concentration of virus used. Despite a highly consistent transduction efficiency (on the basis on eGFP percentages at each dilution for each virus type), the cells demonstrated variable levels of neurotrophin expression.
4.3.3 Neurotrophic Schwann Cells and Mesenchymal Stem Cell Lines

Primary Schwann cells and wild type (non-GFP) MSCs were grown under supernatant media from the established GDNF, NT3 and BDNF retroviral packaging lines for 24 hours prior to antibiotic selection. In the case of the primary Schwann cells, individual colonies became apparent within 4-5 days of growth which became confluent in the flasks by day 12. The cells were then dispersed and either grown for use in scaffolds, or stored at low passage. Figure 4.11 demonstrates DAPI nuclear staining, eGFP fluorescence, and S-100 staining of the NT3 line. Despite some bleaching out of the S-100 staining, there is a homogeneous culture of eGFP positive, S-100 positive cells confirming genetic modification of the glial cell line.
**Figure 4.11:** Schwann Cells genetically modified by pLXSN-NT3-IRES-eGFP retrovirus and selected by G418 antibiotic demonstrate eGFP fluorescence and S-100 staining (TRITC) seen to merge in the composite micrograph. The cells maintain their characteristic spindle morphology. Some bleaching of the S-100 TRITC panel is noted, but cells can still be seen to be positive for the epitope.

Similarly, Schwann Cells infected by GDNF-eGFP retrovirus and subjected to antibiotic selection demonstrate strong eGFP fluorescence as a homogeneous culture, and maintain their characteristic spindle shaped morphology (Figure 4.12).
Figure 4.12: Schwann Cells genetically modified by the pLXSN-GDNF-IRES-eGFP retrovirus demonstrate strong eGFP fluorescence and their characteristic spindle morphology.

Instead of using ELISA, the functional or physiologic capacity of NT3 eluted into the Schwann cell growth media from the modified cell line was assessed with dorsal root ganglia outgrowth experiments. Control media from non-transduced Schwann cells, which contains growth additives Neuregulin and forskolin, did not stimulate neurite outgrowth after 48 hours (Figure 4.13). The extent of neurite outgrowth was seen to be dose dependent at supernatant media dilutions of 1:10, 1:2, and neat media, with robust outgrowth seen in the presence of diluted media containing eluted at NT3.

Experiments are planned to quantitate the amounts of neurotrophin released from OPF scaffolds loaded with transduced Schwann cells and MSCs by
ELISA. This represents an important concept, to identify the elution capacity of the cell-loaded scaffold as opposed to that of the cell culture monolayer.

Figure 4.13: Whole dorsal root ganglia isolated from embryonic day 15 rat pups were plated and grown in the presence of cell media derived from non-transduced primary Schwann cells (0), or dilutions of supernatant media from the NT3-Schwann cell line (0.1 or 1:10 dilution, 0.5 or 1:2, or undiluted neat media). Neurite outgrowth after 48 hours in the presence of the media was seen to be dose dependent, with dense, robust outgrowth stimulated by undiluted media.

A cell line of MSCs genetically modified by the BDNF retrovirus was similarly developed, and was grown to sufficient cell number is to be used in scaffolds. Unfortunately it became apparent that MSCs demonstrated growth arrest and phenotypic differentiation from their norm, when attempts were made to prolong the cell culture as needed for antibiotic selection to form colonies after retroviral infection. The cells became very broad, flat and fan-like in their cytoplasmic structure (Figure 4.14 A). In some cases the cells were seen to fuse to form multi-nucleated cells (Figure 4.14B). In both cases, the nuclear and cytoplasmic architecture was distorted.
Figure 4.14. MSCs were seen to undergo growth arrest and phenotypic changes following prolonged culture conditions required for antibiotic cell selection following retroviral gene modification (20 x magnification)
The overt change in MSC phenotype lead to the decision to switch viral vectors from retrovirus to lentivirus in order to develop neurotrophic MSC lines for \emph{in vivo} use.

### 4.4 Discussion

From the outset of the project the decision was made to develop neurotrophic cell lines by means of retroviral gene transfer. The lab also has expertise with lentiviral vectors. Through Dr. Thomas Ritter and his leadership of the viral vector facility at NUI Galway, we decided to proceed with retroviral vectors with the advantages of stable integration and cell line selection with antibiotics. Dr. Ritter has long-standing experience with the use of these vectors, and very graciously supplied the plasmid and packaging cell line source materials.

In partnership with the Ritter lab a library of neurotrophic expression vectors was developed, which has served as a valuable resource for the REMEDI collaboration. Much effort was put into early verification of sequence accuracy and expression of the neurotrophic factor. In developing the vectors with a PCR based strategy, it was possible to make an intermediate bank of TOPO clones with several different restriction enzyme sites for ease of use and flexibility in the future in other applications. The neurotrophin genes were optimized with the inclusion of Kozak sequences for more efficient ribosomal translation. Several safeguards were taken to minimize the risk of introducing mutations through PCR, including the use of high fidelity DNA polymerase and a PCR kit optimized for GC rich templates. In the case four of the five constructs, no sequence errors were introduced. In the case of the NGF construct, a point mutation resulted in significant protein mis-folding with the substitution of a lysine to proline residue, such that the protein could not be recognized by ELISA. The NGF construct will need to be remade for future use.

The pLXSN-IRES-eGFP vector itself is a tremendous research tool given that cell lines expressing the viral transcript are traceable by the eGFP reporter. It is true to say that a cell expressing eGFP following gene delivery with this construct would also express the neurotrophin. However, one cannot expect a 1:1 stoichiometry in the translation of both proteins despite the fact that the 5’ cistron
and 3’ cistron are from the same mRNA transcript. Internal Ribosomal Entry Sites (IRES) are sequences of mRNA that are capable of binding the ribosome and initiating translation mid-sequence without the need for transcription factors. IRES were initially found in viral sequences. The sequence within our vector is from encephalomyocarditis virus RNA, as was described by originally by Eckard Wimmer (Jang et al., 1988). The sequences were proposed to function as initiator sites for viral protein translation at times when the host cell translation was quiescent or inhibited, thus ensuring ongoing viral propagation. Several sequences were subsequently found in mammalian cells and proposed to act as internal translation initiation sites at times when normal ‘cap-dependent’ initiation was inhibited, such as during mitosis, cell stress, or apoptosis. On detailed review, many of these IRES sequences were found to harbour cryptic splice sites or promoters, providing evidence that the two portions of sequence in this setting may well be differentially regulated (Kozak, 2005). Most of the evidence for cellular IRES activity rests on bicistronic reporter assays, the reliability of which has been questioned given poor experimental controls (Gilbert, 2010). Arguments have been made that the sequence may not be bicistronic at all, that it is monocistronic and the preference for expression is for the first cistron. In our hands, eGFP expression was seen to be slow to come up and was relatively weak.

In this study a wide range of neurotrophin levels in eGFP positive cells was seen. There was no real quantification of the levels of expression from the eGFP cistron. This points to the limitations of using flow cytometry, in that the cells will either be eGFP-positive or not at a given gating threshold. Post-translational processing of the eGFP portion was different to the neurotrophin, most obviously in that the former remained intracellular and the latter needed to be processed for secretion. The efficiency of transfection was also seen to influence the level of expression from the constructs. A poor efficiency (ranging from 30-40%) was seen using expression vector plasmid in 293T cells, and consequently neurotrophic expression was on the order of 0.6 to 1.4 ng per ml. When retrovirus harvested from the mature packaging cell line was used to infect NIH-3T3 cells, neurotrophic expression ranged between 6 and 20 ng per ml. Here there was widest variability with which the neurotrophin was expressed despite a very similar expression level of eGFP across the 3 viruses. It would have been of interest to do RT-PCR quantification of the eGFP and neurotrophin cistrons.
following viral transfection of 3T3 cells for comparison of their relative expression of the IRES construct.

The titre of the retrovirus should impact on the level of gene expression in the target cells. The titre represents the lowest concentration of virus that is still capable of infecting cells. Retrovirus requires the target cell to be in a dividing state in order to integrate its sequences and infect the cell. Two methods of assessing the functional titre of the retrovirus, eGFP transducing units and colony forming units, were used. Similar results were seen with each method of measurement, and similar titres $1 \times 10^6$ range were obtained for each of the 3 retroviruses group. Significant increases in viral titre, an additional of $1 \times 10^4$ cfu, could be achieved simply by concentrating the viral by centrifugal ultrafiltration. A titre of less than $1 \times 10^5$ was considered to be low and to prompt adjustments to the experimental methods. At one point we did try to use PCR-based methods for viral titre whereby a particular sequence such as the $\psi^+$ sequence could be amplified by RT-PCR and compared a standard curve of known sequence quantity. Primers to the $\psi^+$ sequence were made, but the initial experiments were not successful, and would have required significant investment of time to optimize.

Viral titre would depend on several factors acting in a series, starting with the original efficiency of the retroviral expression plasmid transfection to produce the first lower titre virus and then the transducing efficiency of that virus for the final packaging line. The amount of virus present in supernatant media would then depend on the cell density within the flask of a given size, and the amount of time the culture was allowed to produce virus into the media. The application of neat viral media onto 3T3 cells resulted in cell death. Neat virus supernatant was used on both Schwann cells and MSCs, but given the initiation of antibiotic selection within 24 hours of virus application, it is not clear whether the multiplicity of infection was detrimental to cell survival. The missing experiment here was to perform a colony forming unit analysis on both target cell lines. That MSCs exhibited growth arrest and phenotypic change during the cell line selection process was of significant concern. It was not clear whether the cells did not take well to the viral infection, antibiotic selection, or whether the number of cell divisions needed to develop a stable cell line, particularly the numbers needed for scaffold loading, excessively stressed the cells.
FUTURE DIRECTIONS

Physiologic levels of neurotrophin release from target NIH 353 cells and physiologic function of the neurotrophins from transduced Schwann cell supernatant media has been shown. Correct protein folding is evident by ELISA recognition from and physiologic function is seen with neurite outgrowth from DRGs in response to media from NT3-Schwann cells. Target cells have shown 6-20 ng/ml/10^6 cells in neurotrophin release over a 72 hour time period. At the time of thesis submission, we have operated on an additional 60 animals, as per the methods detailed in Chapter 2, implanting genetically modified Schwann cells and MSCs. OPF+ scaffolds were loaded with Schwann cells genetically modified by pLXSN-IRES-eGFP retrovirus for the delivery of NT3, GDNF and BDNF genes (10 animals per group). One animal group of 10 had MSCs genetically modified with BDNF retrovirus. Two additional lentiviral based vectors were developed to deliver NT3 and GDNF genes to MSCs prior to scaffold loading in the remaining 20 animals. Surgical transections were made at the T9 level in female Sprague-Dawley rats, and the scaffolds were implanted. The animals were maintained to the 4 week time point, sacrificed and the spinal cord scaffolds have been dissected free and embedded in paraffin. We are currently in the process of cutting these blocks for immunohistochemical and vessel analysis, which will include a similar approach to the methods detailed in Chapter 3. The analysis will also build on characterizing the immune response by detailing more precisely what cell subtypes are involved, particularly the macrophage reaction. Furthermore, a group of animals has been completed with scaffold harvest at weekly intervals for four weeks. The analysis here will provide insight into the timing and distribution of cellular migration into the scaffold and the fate of implanted cells.

OPF scaffolds clearly have the capability to integrate a combination therapies for spinal cord repair. While using OPF+ scaffolds to house various cell lines, I would like to further develop OPF as a mean to deliver therapeutic molecules from the polymer itself. I have particular interest in developing an OPF co-polymer with fibrin. Embedding fibrin into the OPF hydrogel may allow for the controlled release of nucleic acid transfection polyplexes (Kulkarni et al., 2009) to deliver plasmid vectors or siRNA, or for the release of virus for gene delivery (Breen et al., 2009). Polymeric delivery of chondroitinase ABC for
glycosaminoglycan digestion, either from the hydrogel itself through charge modification (Dadsetan et al.) or by viral elution from a fibrin co-polymer, is an essential next step (Garcia-Alias et al.; Zhao et al., 2011). The model itself in this context could be extended to study astrocyte-Schwann cell interactions in vivo as well as plasticity of axonal growth given neurotrophic cues and proteoglycan degradation. Finally I am interested in extending a clinical analysis of the animals, to include further use of nerve conduction studies and MRI imaging. Applications of MRI axon tractography may enable tracking of axon extension through the grafts while the animals are alive.
Table 2.1

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue #</th>
<th>Reactivity</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD73 (MSC) (+)</td>
<td>Ms IgG1 k</td>
<td>BD Pharmingen</td>
<td>551123</td>
<td>rat monoclonal</td>
</tr>
<tr>
<td>CD90 (MSC, fibroblasts) (+)</td>
<td>Ms IgG1 k</td>
<td>Biolegend</td>
<td>202501</td>
<td>rat, mouse</td>
</tr>
<tr>
<td>CD11b/c (macrophages) (-)</td>
<td>Ms IgG2a k</td>
<td>Biolegend</td>
<td>201801</td>
<td>rat monoclonal</td>
</tr>
<tr>
<td>CD172 SIRP (myeloid lineage) (-)</td>
<td>Ms IgG2a k</td>
<td>BD Pharmingen</td>
<td>552297</td>
<td>rat monoclonal</td>
</tr>
<tr>
<td>CD71 (transferring receptor) (+/-)</td>
<td>Ms IgG2a k</td>
<td>BD Pharmingen</td>
<td>554890</td>
<td>rat monoclonal</td>
</tr>
<tr>
<td>CD34 (haemapoetic stem cells) (-)</td>
<td>Goat IgG</td>
<td>R&amp;D</td>
<td>AF4117</td>
<td>rat monoclonal</td>
</tr>
</tbody>
</table>

Listed are antibodies used for flow cytometry analysis of mesenchymal stem cell surface markers.

Table 2.2

<table>
<thead>
<tr>
<th>RECIPE for Positively Charged OPF</th>
<th>Neutral</th>
<th>20% Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPF (g)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>I-2959 photoinitiator (ul)</td>
<td>375</td>
<td>375</td>
</tr>
<tr>
<td>MEATAC charge (ul)</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>Water (ul)</td>
<td>475</td>
<td>325</td>
</tr>
<tr>
<td>NVP cross-linker (ul)</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td><strong>Total (ul)</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
</tr>
</tbody>
</table>

Listed are the relative proportions of OPF, photoinitiator, NVP, MAETAC and distilled water to form the neutral and positively charged hydrogel. The recipe can be halved or quartered as the experiment demands. The solution was made fresh for each application.
<table>
<thead>
<tr>
<th>Score</th>
<th>Range of Locomotor Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No hindlimb (HL) movement observed</td>
</tr>
<tr>
<td>1</td>
<td>Slight movement of 2 joints (usually hip &amp;/or knee joint)</td>
</tr>
<tr>
<td>2</td>
<td>Extensive movement of 1 joint with/without slight movement of another joint</td>
</tr>
<tr>
<td>3</td>
<td>Extensive movement of 2 joints</td>
</tr>
<tr>
<td>4</td>
<td>Slight movement of all 3 HL joints</td>
</tr>
<tr>
<td>5</td>
<td>Slight movement of 2 joints with extensive movement of the 3rd</td>
</tr>
<tr>
<td>6</td>
<td>Extensive movement of 2 joints with slight movement of the 3rd</td>
</tr>
<tr>
<td>7</td>
<td>Extensive movement of all 3 HL joints</td>
</tr>
<tr>
<td>8</td>
<td>Sweeping or plantar placement of paw with no weight support</td>
</tr>
<tr>
<td>9</td>
<td>Plantar placement of paw with weight support while stationary, or occasional, frequent or consistent weight-supported dorsal stepping &amp; no plantar stepping</td>
</tr>
<tr>
<td>10</td>
<td>Occasional weight-supported plantar stepping but no Forelimb (FL)-HL coordination</td>
</tr>
<tr>
<td>11</td>
<td>Frequent-consistent weight-supported plantar steps &amp; no FL-HL coordination</td>
</tr>
<tr>
<td>12</td>
<td>Frequent-consistent weight-supported plantar steps &amp; occasional FL-HL coordination</td>
</tr>
<tr>
<td>13</td>
<td>Frequent-consistent weight-supported plantar steps &amp; frequent FL-HL coordination</td>
</tr>
<tr>
<td>14</td>
<td>Consistent weight-supported plantar steps, consistent FL-HL coordination &amp; predominant paw position during locomotion is rotated when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL-HL coordination &amp; occasional dorsal stepping</td>
</tr>
<tr>
<td>15</td>
<td>Consistent plantar stepping &amp; consistent FL-HL coordination &amp; no/occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body of initial contact</td>
</tr>
<tr>
<td>16</td>
<td>Consistent plantar stepping &amp; consistent FL-HL coordination during gait &amp; toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at the initial contact &amp; rotated at the lift off</td>
</tr>
<tr>
<td>17</td>
<td>Consistent plantar stepping &amp; consistent FL-HL coordination during gait &amp; toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at the initial contact &amp; lift off</td>
</tr>
<tr>
<td>18</td>
<td>Consistent plantar stepping &amp; consistent FL-HL coordination during gait &amp; toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at the initial contact &amp; rotated at the lift off</td>
</tr>
<tr>
<td>19</td>
<td>Consistent plantar stepping &amp; consistent FL-HL coordination during gait, toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at the initial contact &amp; lift off, &amp; tail is down part or all of the time</td>
</tr>
<tr>
<td>20</td>
<td>Consistent plantar stepping &amp; coordinated gait, consistent with toe clearance, predominant paw position is parallel at initial contact &amp; lift off, &amp; trunk instability; tail is consistently up</td>
</tr>
<tr>
<td>21</td>
<td>Consistent plantar stepping &amp; coordinated gait, consistent with toe clearance, predominant paw position is parallel throughout stance, &amp; consistent &amp; trunk stability; tail is consistently up</td>
</tr>
</tbody>
</table>

**Table 2.3: The Basso, Beattie and Bresnahan (BBB) locomotor rating scale**

Taken from Basso, et al., 1995.
Table 2.4. Summary of the peri- and post operative evaluations for Control and Schwann cell animals.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Group</th>
<th>Polymer Type &amp; Channel Size</th>
<th>Cell Number per ml</th>
<th>Cell Number per Scaffold</th>
<th>Wt. before Surgery</th>
<th>Surgery Date</th>
<th>Aftercare Observations</th>
<th>Autophagia Score</th>
<th>Days on Buprenex</th>
<th>Days on Abx</th>
<th>Perfusion Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM001C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/16/2008</td>
<td>Lung Puncture 17/12, by 2nd housed rat</td>
<td>0.67</td>
<td>9.00</td>
<td>14.44</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM002C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/16/2008</td>
<td>haem cyst at incision site</td>
<td>1</td>
<td>6</td>
<td>14</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM003C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/16/2008</td>
<td>Front paw autophagia</td>
<td>2</td>
<td>17</td>
<td>8</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM004C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/16/2008</td>
<td>Front paw autophagia</td>
<td>0</td>
<td>6</td>
<td>8</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM005C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Injection site problems</td>
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<td>11</td>
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<td>1/15/2009</td>
</tr>
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<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Injection site problems</td>
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<td>11</td>
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<td>1/15/2009</td>
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<td>OPF+</td>
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<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Injection site problems</td>
<td>1</td>
<td>11</td>
<td>18</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM008C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Back paws, collar</td>
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<td>7</td>
<td>14</td>
<td>1/15/2009</td>
</tr>
<tr>
<td>NM009C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Died sepsis 23/12</td>
<td>0.67</td>
<td>9.00</td>
<td>14.44</td>
<td>1/15/2009</td>
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<tr>
<td>NM010C</td>
<td>Control</td>
<td>OPF+</td>
<td>Matrigel</td>
<td>Matrigel</td>
<td>50,000</td>
<td>12/17/2008</td>
<td>Died sepsis 27/12, opened up own belly</td>
<td>0.57</td>
<td>6.00</td>
<td>20.17</td>
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<tr>
<td>NM011S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>230</td>
<td>12/18/2008</td>
<td>0</td>
<td>6</td>
<td>20</td>
<td>1/15/2009</td>
<td></td>
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<td>NM012S</td>
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<td>OPF+</td>
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<td>400,000</td>
<td>240</td>
<td>12/18/2008</td>
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<td>6</td>
<td>19</td>
<td>1/15/2009</td>
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<td>50,000</td>
<td>400,000</td>
<td>230</td>
<td>12/18/2008</td>
<td>0</td>
<td>6</td>
<td>24</td>
<td>1/15/2009</td>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>230</td>
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<td>6</td>
<td>21</td>
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<td>NM015S</td>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>230</td>
<td>12/18/2008</td>
<td>0</td>
<td>6</td>
<td>14</td>
<td>1/15/2009</td>
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<td>NM016S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>245</td>
<td>12/18/2008</td>
<td>0</td>
<td>6</td>
<td>23</td>
<td>1/15/2009</td>
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<tr>
<td>NM017S</td>
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<td>OPF+</td>
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<td>400,000</td>
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<td>20.17</td>
<td>1/15/2009</td>
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<tr>
<td>NM018S</td>
<td>Schwann cell</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>12/18/2008</td>
<td>0</td>
<td>6</td>
<td>23</td>
<td>1/15/2009</td>
<td></td>
<td></td>
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<tr>
<td>Animal ID</td>
<td>Group</td>
<td>Polymer Type &amp; Channel Size</td>
<td>Cell Number per ml</td>
<td>Cell Number per Scaffold</td>
<td>Wt. before Surgery</td>
<td>Surgery Date</td>
<td>Aftercare Observations</td>
<td>Autophagia Score</td>
<td>Days on Buprenex</td>
<td>Days on Abx</td>
<td>Perfusion Date</td>
</tr>
<tr>
<td>-----------</td>
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<td>----------------</td>
<td>----------------</td>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>NM019M</td>
<td>MSC (poor growth in culture)</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>250</td>
<td>12/18/2008</td>
<td>injection site problems</td>
<td>0</td>
<td>7</td>
<td>23</td>
<td>1/26/2009</td>
</tr>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>250</td>
<td>1/19/2009</td>
<td>died post op (Sr anaesthesia)</td>
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<td>7</td>
<td>22</td>
<td>1/26/2009</td>
</tr>
<tr>
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<td>MSC (poor growth in culture)</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>245</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
<td>2</td>
<td>8</td>
<td>24</td>
<td>3/26/2009</td>
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<tr>
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<td>MSC</td>
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<td>400,000</td>
<td>245</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
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<td>8</td>
<td>23</td>
<td>3/26/2009</td>
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<tr>
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<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>245</td>
<td>2/24/2009</td>
<td>died intra op. bleeding</td>
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<td>8</td>
<td>23</td>
<td>3/26/2009</td>
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<tr>
<td>NM024M</td>
<td>MSC</td>
<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>250</td>
<td>2/24/2009</td>
<td>Scaffold taken back out from RIP animal</td>
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<td>4</td>
<td>4</td>
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</tr>
<tr>
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<td>MSC</td>
<td>OPF+</td>
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<td>400,000</td>
<td>250</td>
<td>2/24/2009</td>
<td>Euthanasia, back leg autophagia</td>
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<td>20</td>
<td>3/26/2009</td>
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<td>400,000</td>
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<td>32</td>
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<td>NM027M</td>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>230</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
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<td>8</td>
<td>26</td>
<td>3/26/2009</td>
</tr>
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<td>NM028M</td>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>270</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
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<td>26</td>
<td>3/26/2009</td>
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<tr>
<td>NM029M</td>
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<td>OPF+</td>
<td>50,000</td>
<td>400,000</td>
<td>230</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
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<td>8</td>
<td>26</td>
<td>3/26/2009</td>
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<tr>
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<td>400,000</td>
<td>230</td>
<td>2/24/2009</td>
<td>front paw autophagia</td>
<td>0</td>
<td>6</td>
<td>26</td>
<td>3/26/2009</td>
</tr>
</tbody>
</table>

1.00 7.78 24.33
APPENDIX, CHAPTER 3:
Antibodies used for immunohistochemistry

Antibody specificity was a concern given the use of heat-induced epitope retrieval from paraffin embedded tissue. To date the laboratory has not used frozen tissue preparations, as it was thought necessary to fixate the scaffold structure rigidly in paraffin to enable microtome sectioning. Primary antibodies that had recommendations for the use in paraffin embedded tissue were sourced.

**DAKO:**

*Rabbit Anti-Glial Fibrillary Acidic Protein*, code No. Z0334 can be applied on formalin-fixed, paraffin-embedded sections of human brain following antigen retrieval; (http://www.dako.com/us/download.pdf?objectid=104722002), and as applied in (Hol et al., 2003) at a dilution of (1:300), and (Lyck et al., 2008) at a dilution of 1:2000. This antibody was used for standardization of immunohistochemical procedures in the Nordic immunohistochemical Quality Control (NordiQC) collaboration. The antibody was also for standardization of immunohistochemical procedures in UK National External Quality Assessment Scheme for Immunocytochemistry (UK NEQAS). (Lyck et al., 2008) This GFAP antibody shows 90-95% homology between species including the rat (Castellano et al., 1991).

*Monoclonal Mouse Anti-Human Neurofilament Protein*, code No. M0762, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections. Pre-treatment of tissues with heat-induced epitope retrieval is recommended, as per application in (Henwood, 2010), and http://www.dako.com/us/download.pdf?objectid=104933003.

**BD PHARMINGEN:**

*Purified Mouse Anti-Rat CD45R* (catalogue 554879) is referenced and reported for the application in immunohistochemistry with formalin-fixed tissue requiring antigen retrieval in (Kroese et al., 1990), and as per the data sheet: http://wwwbdbiosciences.com/external_files/pm/doc/tds/rat/live/web_enabled/22161D_554879.pdf
Purified Mouse Anti-Vimentin antibody (catalogue 550513) has similarly been reported for use in paraffin embedded tissue (Cokakli et al., 2009) and (Kim et al., 2007), and as per:


ABCAM:
Anti-collagen IV rabbit polyclonal antibody (Ab6586) has been used in several publications in paraffin embedded rat tissue (Hornigold et al.), (Barnes et al., 2011), and http://www.abcam.com/Collagen-IV-antibody-ab6586-references.html

WAKO:
For the anti-Iba1 rabbit polyclonal antibody (catalogue 019-19741), the company provides a protocol specific to paraffin embedded sections: http://www.wako-chem.co.jp/english/labchem/product/life/AntiIba1/pdf/leaf.pdf. The antibody has been reported for use in rats in paraffin sections (Henriksson and Tjalve, 2000).

SEROTEC:
Mouse anti rat monoclonal CD3 antibody (IgM) (MCA772) is recommended for use in paraffin sections using antigen retrieval methods (dilution 1:10), (McKechnie et al., 1997), and in:


BIOGENEX:
Mouse anti S-100 Protein (clone 15E2E2) (catalogue MU058-UC) is a monoclonal antibody designed specifically for paraffin embedded tissue, http://www.biogenex.com/3291, and as published in (Feldman et al., 2008).
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Cat #</th>
<th>Reactivity</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b(^1)</td>
<td>Ms IgG</td>
<td>Chemicon</td>
<td>CBL1512</td>
<td>Rat</td>
<td>100</td>
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<tr>
<td>GFAP</td>
<td>Rabbit polyclonal</td>
<td>Dako</td>
<td>Z0334</td>
<td>Rat, mouse</td>
<td>100</td>
</tr>
<tr>
<td>NG2</td>
<td>Ms IgG</td>
<td>Chemicon</td>
<td>MAB5520</td>
<td>Human</td>
<td>100</td>
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<td>Vimentin</td>
<td>Ms IgG1</td>
<td>BD Pharmingen</td>
<td>550513</td>
<td>Human, Rat, Mouse</td>
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<tr>
<td>Collagen IV</td>
<td>Rabbit Polyclonal</td>
<td>AbCam</td>
<td>Ab6586</td>
<td>Human, Rat, Mouse</td>
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**MAYO CLINIC**

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Cat #</th>
<th>Reactivity</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurofilament</td>
<td>Ms IgG1 k</td>
<td>Dako</td>
<td>M0762</td>
<td>human clone 2F11, rat</td>
<td>50-100</td>
</tr>
<tr>
<td>S-100</td>
<td>Ms IgG2a k</td>
<td>Biogenex</td>
<td>MU058-UC</td>
<td>human clone 15E2E2, rat</td>
<td>300-400</td>
</tr>
<tr>
<td>CD45R (B-Cells)</td>
<td>Ms IgG2b k</td>
<td>BD Pharmingen</td>
<td>554879</td>
<td>rat monoclonal</td>
<td>50</td>
</tr>
<tr>
<td>CD68 (macrophages)(^1)</td>
<td>Ms IgG1 k</td>
<td>Serotec</td>
<td>MCA341R</td>
<td>rat monoclonal</td>
<td>100</td>
</tr>
<tr>
<td>CD3 (T-Cells)</td>
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<td>MCA772</td>
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<td>Rabbit Poly</td>
<td>Wako</td>
<td>019-19741</td>
<td>Human, rat, mouse</td>
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</table>

\(^1\) CD68 and CD11b antibodies were not used given superiority of the Iba1 antibody.
### TABLE 3.2 Secondary Antibodies used for Immunohistochemistry

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<th>Secondary Antibody</th>
<th>Labelled</th>
<th>Species</th>
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<th>Optimal Dilution</th>
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<td></td>
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<td>Anti Ms IgG</td>
<td>FITC</td>
<td>Goat</td>
<td>Molecular Probes</td>
<td>A11001</td>
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<td>Goat</td>
<td>Chemicon</td>
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<td>Goat</td>
<td>Sigma</td>
<td>T5393</td>
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<tr>
<td>Anti Mouse IgM (μ chain)</td>
<td>FITC</td>
<td>Goat</td>
<td>Sigma</td>
<td>F9259</td>
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<td>AbCam</td>
<td>ab6717</td>
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<td>Chemicon</td>
<td>AP181S</td>
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<td>Molecular Probes</td>
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<td><strong>MAYO CLINIC</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti Rabbit IgG</td>
<td>Biotin</td>
<td>Goat</td>
<td>Dako</td>
<td>E0432</td>
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<td>Biotin</td>
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<td>Cy-2</td>
<td>Goat</td>
<td>Jackson Laboratories</td>
<td>115-225-166</td>
<td>150</td>
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<tr>
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<td>Goat</td>
<td>Jackson Laboratories</td>
<td>115-165-166</td>
<td>150</td>
</tr>
<tr>
<td>Anti Mouse IgM-HRP conjugate, rat absorbed</td>
<td>HRP</td>
<td>Goat</td>
<td>Serotec</td>
<td>STAR86P</td>
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<td>Goat</td>
<td>Serotec</td>
<td>STAR77</td>
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</tbody>
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APPENDIX, CHAPTER 4:

Human Neurotrophin Sequences and Primers used for Amplification and Sequencing

NT-3

Nucleotide Sequence (774 nt):

ATGTCCATCTTTGTATTTGATATTTCGCTTTATCTCCGTGGCATC
GGTACCAACATGGATCAAAGGAGTTTGCGCAGAAGACTCGTCAATTCC
ACATTATAGCTGATCCAGCAGATATTATTGAAAAACAAGCTCTC
GCAAGATGGTGAGCTTAAGGAATAATTACCAGAGCACCTGGCCAAAGC
TGAGGCTCCCGAGACCGGGAGGCGGGAGGCGCCGCAAGTCAGCAT
CCAGCGGTATGCAATGGACACCGAAGACTCGTGGCAAAGAGAGC
CTACAACTCAACCGGGTCTCTCGTGACGCAGACGACCCCTTTGGAGCC
CCGCCCTTGTATCTCATAGGAGGATTACGTGGGCGAGCCCGTGGTG
ACACAACATCACGGCGAAACGTTAGCAGGACAAAAAGTGATCAGC
GGGAGTACTCGGTATGTGAGCTGAGAGTCTGTGTTGACGGCAAGAGC
ATCAGCCATCAGACATTCCGGGGACACCAGGCAGACCGTGGCGAGGA
GTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG
ATCAAAACGGCCACATCCTCCGTCAAAACATATATATATATATATATAT
TAAGGAAGCCAGGCCGCAAAAACCGGTGACGGGTTGACGGGTTGACGG
CAGGAGACCGGGCTCAAACTCGGACGGGCTCGGACGGGCTCGGACG
CTCCAGTAGAAGAAAAGCTCGTGGGCGGCTGAGGATACGGTACATG
AGGTGCAAAAGATCGGAGAAGAACATGA

Translation (257 aa):

MSILFYVFILARLGICQUGNMDQRSLPSDLSNSIIKLIQADILKNKLSDKMV
DVKENYQSTLPKAEAPREPERGGPAKSAFQPVIAMTDELRQQRRYNSPRV
LSSDSTPPLLPMEMYVSGPVANRTSRKRYAEHSRGEYSVCDSE
SLWVTDKSSADIRGQHTVLEIIGKTGNSPVQFYETRCKEARPVKNCR
GIDDKHWNSQCKTSQTYVRALTSNKLVGVWRIRSDVCALSKRIGRT

Amplification Primers for Cloning:

5'--> 3' Forward

EcoRI Kozak 18 bp
gGAATTC GCCACC ATGTCCATCTTTGTATTTTAT (31 bp)

MluI
ACGCGT GCCACC ATGTCCATCTTTGTATTTTAT (30 bp)

3'--> 5' (Reverse compliment of AAAATCGGAAGAA

Sall 18 bp
GTCGAC TCATGTTCTCCGATTTT (24 bp)
Axonal Regeneration Supported by OPF+ Cell-loaded Hydrogel Scaffolds

CNTF

Nucleotide Sequence (603 nt):

ATGGCTTTTCACAGACATTCACCCCTCACCCTGGGACCTCTGAGCCCGCTTATCCAGCATAGATCGGCTGGACAAGCTGACTCTTACGGAATCCTATGTGAAGCATCAGGGCCTGAGAACCTCATCAGTTACCTTCCATGTTTTGTTGGCCAGGCTCTTAGAAGACCAGCAGGTGCATTTTACCCCAACCAGAAGGTGACTTCCATCAAGCTATACATACCCTTCTTCTCAAGTGCCTGGCTTTTGCATACCCAGATAGAGGAGATTATGATACTCCTGGAATACAAGATCCCCCGCAATGAGGCTGATGGGATGCCTATTAATGTTTGAGAATGGTGCTCTTGGAGATGGTGGTCTCTTTGAGAAGAAGCTGTGGGGCCTAAAAGGTGCTGCAGGAGCTTTCACAGTGGACAGTAAGGTCCATCCATGACCTTCGTTTCATTTCTTCTCATCAGACTGGGATCCCAAGCAGCGTGGGAGCATATATTGCTAACAAGAAAAATGTAG

Translation (200 aa):

MAFTEHSPLTPHRRDLCSRSIWLARKIRSDLTALTESYVHKHQGLKNKINLDSDGMPVASTDQWSELTEAEQLQENLQAYRTFHVLLARLLEDQQVHFTPTEGDHFQAIHTLQLQVFAAYQIEELMILLEYKIPRNEADGMPINVGDGGLFEKKLWGLKVLQELSQWTVRSHDLRFISSHQTGIPARGSHYIANNKKM

Amplification Primers for Cloning:

5'--> 3' Forward

EcoRI Kozak 18 bp
gGAATTC GCCACC ATGGCTTTTCACAGACAT (31 bp)

MluI
ACGCGT GCCACC ATGGCTTTTCACAGACAT (30 bp)

3'--> 5' (Reverse, compliment of AACAACAAGAAAATGTAG)

SalI 18 bp
GTCGAC CTACATTTTCTTGGT (24 bp)
**b-NGF**

Nucleotide Sequence (726 nt):

```
ATGTCCATGTGGTTCTACACTCTGATCACAGGCGATAC
AGGGGCGAACCACACTCAAGAGCAATGTCCTGITTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACCTGCCCCTTCC
CACAGGCGCAAGGCGCAGCGCCCCGCAGCGCAGCGGCGAGCGCAGCGGCGCAGCGGCG
GGGCGAGAACCACACTCAAGAGCAATGTCCTGITTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCCCCAGCCCCACTGGGACTAAACTTCAGCATTTCCCTTTGACGACCACCACACCAC
CCC
```

Translation (241 aa):

```
MSMLFYTLITAFILGIGQIAEPHSESINVPAGHTIPQAHTKLQHSLDTRRA
RSAPAAAIAARVAGQTRTNITYDPRLFHKRRRLSPVRFLSTQPRAAADTQD
LDFFEVGGAAPFNRTHRSKRSSSHIPFHRGEFVSDSVSVWVGDKTTATDI
KGKEVMPVGLEVNINNSFQKYFPETKCRDPNPVDSGCRGIDSKHWSYCT
TTHTHVFKALTDMDGKAAWRFIRIDTACVCLSRSKAVRRA
```

**Amplification Primers for Cloning:**

5'--> 3' Forward

- **SalI** Kozak 18 bp
  - acgcGTGAC GCCACC ATGTCCATGTTGTTCTAC (34 bp)

- **BamHI**
  - GGATCC GCCACC ATGTCCATGTTGTTCTAC (30 bp)

3'--> 5' (Reverse, compliment of GCTTGAGAAGAGCCTGA)

- **SalI** 18 bp
  - GTGAC TCAGGCTTCTCTCACAGC (24 bp)
**BDNF**

Nucleotide Sequence (743 bp)

```
ATGACCATCCTTTT CCTTACTATGTTATTTCCTACTTT GGTG CATGA
AGGCTGCCAATGAAAGAAGCAAACATCCGAGGACAAAGGTTGGCTTG
GCCTACCCAGGTGTGCGGACCATGGGACTCTGGAGAGCCGAATGCA
GCCCAAGGCGAGGGTTCAAGAGGCTTTGCACATCTATTGCTGAGACT
ACACGCTGATAGAACAGCTGGTGGATGAGGACCAGAAAGTTTCGGCCCA
ATGAGAAGAAATATTAAAGGAGCAGCAGACTTGGTACCTCCAGGGTGATG
CTCACTAGTCAAGTCCTTTGGAGCCCTCTTCTTTCTCTTCTGGCTGAGG
AATACAAAATTACCTAGATGCTGCAAACATGCTCCTAGGAGTCCCGGC
GCCACTCTGACCCTGCCCGCCGAGGGAGGCTGAGCTGAGCTGACAGTA
TTAGTGAGTGGTAAACGGCGCGAGCACAAGAAGACTGCAAGTGACATG
TCGCCGCGGAGGTCACAGTCTCTTGAAAGGTCCTGCTTACAAAAAGGC
CAACTGAGCAATCTCTTACGAGACCAAGTGAATCCCACAGGAGTTAC
ACAAAAAGGAGGGCTGAGGCGGCTAGACAAAGGCACTTGGCAGACTCCA
GTGCCGGACACTCCCGTGCTAGCTGGGCTGCCCTTGACATTGGAATAGCA
AAAGGAGAAATGGCTGCGGATTTCAAGGAGATGGACTCTTCTGGTATG
TACATTGCCAATAAAAAGGGAAGGATAG
```

Translation (247aa)

```
MTILFLTMVISYFGCMKAAPMKEANIRGQGGLAYPGVRTHTGTE
SVNGPKAGRSRGLTSLADTFEHVIEELLDQKVRPNEENNKDADLYTSRV
MLSSQVPL
EPPLLFFLEEYKNYLDANSMRVRHSDPARRGELSVCDSISEWVTAADKKTAVDMS
GGTVTVLEKVPVSKQGLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCRTTQSYVRAL
```

**Amplification Primers for Cloning:**

5'----> 3' Forward

MluI Kozak 18 bp
ACGGCGT GCCACC ATGACCATCCTTTTCTTT

3' ----> 5' (Reverse complement of ACCATTTAAGGGGAAGATAG)

SalI 18 bp
GTCGAC CTATCTCCCTTTTAAT
Axonal Regeneration Supported by OPF+ Cell-loaded Hydrogel Scaffolds

GDNF

Nucleotide sequence (635 bp)

ATGAAGTTATGGGATGTCGTTGCCTGCTGCTGTGCTGCACTCCACCACC
GCCGCGCCCTCCTGCGTGCGCGCGGCTACAGGAGCCCTCGCAGGCGAGG
GCCGAGACCGCCTCCCTCGGCCGCGCGCGCGCGGCTGGCTGCTGAGC
AGTGAATGAAATGCGAGAGGATTATACCTGAGTTCTGAGATGATGTC
ATGGAATATTTATTTCAAGCCACCCATTAAAGACTGAAGAGTCAACCAGAT
AACAAATATGCAGTGGCTTCTGAGAAGAGGCAGCGAGCGAGAGTACGCTG
AGCTGCAACCAGAAATACCGAGAGAGGAAGGTCGAGAGGCGACAA
GGCGCAAAAACCAGGGGTTGTGCTCTTAAACTGCAACCATATTTAAATG
CTCACTGATTGGTCCTGGCTATGAGAACCAGGAGGAAGGACTGATTATTTT
AGTAGTGAGAGTCAACCAGGCGGCCGCCGCCGACCCAGAGAATTCCAGAG
GGAAAAGGTCGGAGAGGCCAGAGGGGCAAAAACCGGGGTTGTGCTTAA
CTGACTTGGGTCTGGGCTATGAAACCAAGGAAGGAACTGAGATTTT
TAGATATTTTAACCATATCTTGAAGAAGAGGATCCGAGAGGCGACAA
ATGGAATATTTTACCATATCTTGAAGAAGAGGATCCGAGAGGCGACAA
GTGAGATGATATCTGA

Translation (211 aa)

MKLWDVVAVCLVLLHTASAFPLPAOGKRPPEAPAEEDRSLGGRRRAPFALSS
DSNMPEDYPDQFDVDIFQATIKRLKRSVPDKQMAMVRPRERNRQAAA
NPENSRKGRGRQRGRGKRNRCVLTAIHLNVLDGLGYETKEELIFRYSGS
CDAAETTDLKILKNLSRNRLVSDKVQACCRPIAFDDDLSFLDDNLVYH
ILRKHSARCKGCI
TMDSKKRRGWRFIRIDTSCVCTLTIKGR

**Amplification Primers for Cloning:**

5' ----> 3' Forward

MluI Kozak 18 bp
ACGCGT GCCACC ATGAAGTTATGGGATGTC (GDNF variant 1)

3' ----> 5' reverse complement of AGGTGTGGATGTATCTGA (variant 1)

SalI 18 bp
GTCGAC TCAGATACATCCACACCT

**pLXSN-IRES-eGFP Primers for Neurotrophin Insert Sequencing**

5' → 3'
GGCGCGGGAATTCGCCGCTCTGCTGCTGCTGCTG (bp 1462 - 1480)

3' → 5'
ACCGACAAGTCATCGGCG (bp 1971-1990)
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