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Peripheral Nerve Regeneration: A Study of Surgical and Biological Techniques to Enhance Functional Regeneration

by

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Abstract

Unlike the central nervous system, axonal regeneration does occur in the peripheral nervous system, however, despite this, functional recovery from nerve transection injury remains dismal. This has been attributed to factors intrinsic to the motor or sensory cell body and to elements in the local site of injury including nerve gaps, scar and a limited time frame in which supportive growth factors and extracellular matrix molecules are expressed. The aim of this thesis is to review the mechanisms behind axonal damage and regeneration in the peripheral nervous system and investigate surgical, pharmacological and biological approaches to overcome limitations in regeneration and functional recovery. By taking a broad approach to the topic, I hoped to gain a greater understanding of the inhibitory and regenerative processes at play and provide a contribution to the understanding in the field of peripheral nerve surgery.

Acknowledgement

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Abbreviations

BME	β-mercaptoethanol
BMSSC	Bone marrow stromal stem cells
BDNF	Brain derived neurotrophic factor
CAM	Cell adhesion molecule
CFU	Colony forming unit
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CGRP	Calcitonin gene related peptide
CSPG	Chondroitin sulfate proteoglycan
DMSO	Dimethylsulfoxide
DRG	Dorsal root ganglion
ECM	Extra-cellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GAP	Growth associated protein
G-CSF	Granulocyte colony stimulating factor
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMF	Glial maturation factor
HRG	Heregulin
HSPG	Heparin-sulfate-proteoglycan
IFN	Interferon
IGF	Insulin like growth factor
IL	Interleukin
LIF	Leukocyte inhibitory factor
MAPC	Multipotential adult progenitor cells
MIAMI	Marrow isolated adult multilineage inducible cells
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
NT	Neurotrophin
PNS	Peripheral nervous system
PSA	Polysialic acid
PDGF	Platelet derived growth factor
RA	Retinoic acid
RS	Recycling stem cells
SC	Schwann cell
SCF	Stem cell factor
SFI	Sciatic nerve function index
SPC	Stromal precursor cells
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VIP	Vasoactive intestinal peptide

Chapter 1: Pathophysiology of Nerve Injury and Current Approaches to Nerve Repair

Introduction

Treatment of injuries to the nervous system, both central and peripheral, is a challenge due to the inherent complexity and topographic arrangement of nerve tissue. In addition, the limited regenerative capacity of the peripheral nervous system (PNS) and lack of regenerative potential in the central nervous system makes these injuries difficult to treat. Over the years many technical advances have improved regeneration in the PNS, most notably the advent of microsurgical anastomosis. Despite these advances functional recovery following injury to the nervous system remains poor. Recent research has focused on a biological approach to healing, looking at growth factors and stem cells in order to promote an environment conducive to growth, functional regeneration and recovery. Among the most exciting areas of research is the discovery of mesenchymal stem cells (MSCs) and their inherent plasticity allowing for differentiation into multiple cell lineages including chondrocytes, osteocytes, adipocytes and neuronal-like cells. This has led to the investigation of stem cell targeting of injured tissues. Much remains to be elucidated including the characterization of MSCs, the derivation of an adequate delivery method and the evaluation of the effect and mechanism of action of stem cells on injured tissue. Research examining the effect of stem cells on the central and more recently the PNS has led to the discovery of many growth factors and extracellular matrix proteins that have been shown to be essential to regeneration. The specific role of MSCs in nerve injury

has also been questioned. Do they exert their effect structurally by differentiating into myelinating Schwann cells or do they exert a neurotrophic effect through growth factor elaboration? Do they play a role in the recruitment of neurotropic extracellular matrix proteins? Even the basic question of whether the *exogenous* application of MSCs to peripheral nerve injuries has a beneficial effect on regeneration has generated conflicting evidence.

The purpose of this paper is to review the current knowledge in nerve injury biology and pathophysiology, and the potential role of MSCs in the treatment of peripheral nerve injuries.

Molecular Mechanisms of Nerve injury

Neuronal response to injury in the nervous system can be divided into two broad categories: survival and regeneration. Following injury, an injured neuron will either undergo apoptosis or regenerate. There exist three areas of active response in the injured nerve: the cell body, the proximal axon stump and the distal axon stump. The combined responses of these areas guide the regenerative process and dictate the fate of the injured neuron.

Cell Body Response to Injury

The neuron cell body responds to injury to the axon by undergoing characteristic changes that include neuronal nuclear eccentricity, nucleolar swelling and dissolution of Nissel bodies, a process termed chromatolysis. The

neuron may undergo apoptosis and if it survives, the cell body reaction leads to axon regeneration. Survival of a neuron is the first pre-requisite for regeneration and depends on several factors including neuron type, age of patient, extent of injury and the proximity of axon injury to the cell body. Motor neurons are less susceptible to death than sensory neurons. Mature neurons in adults are less susceptible to death than immature neurons in the developing animal. Injuries to neurons far from the cell body are less likely to induce cell death than injuries close to the cell body [63]. The actual mechanism that determines whether an injured nerve undergoes apoptosis or regeneration is still unknown and remains an active area of research. Neurotrophic factors have been implicated in preventing apoptosis. Studies in which the distal nerve stump has been sequestered have shown an increase in neuronal cell death [118]. However, the distal nerve stump is not the only source of neurotrophic support as axotomized neurons can survive in absence of the distal stump for periods greater than one year[207]. This additional trophic support may be derived from glial cells within the spinal cord or neurotrophic factors produced by the neurons themselves. Many neurotrophic factors have been implicated in promoting axon regeneration including brain derived neurotrophic nerve factor (BDNF) and nerve growth factor[112,113]. If neurons survive the injury, the axotomized neuron assumes a regenerative phenotype of active proliferation and growth factor elaboration. Surviving neurons increase production of tubulin, actin, calcitonin gene-related peptide (CGRP), growth associated protein 43 (GAP-43) and other growth associated proteins[63,198,199].

Although axotomy is associated with down-regulation of motor, sympathetic and sensory neurotransmitters, there are several signaling neuropeptides that are upregulated[75,228]. In axotomized motoneurons, CGRP is upregulated, which is thought to sustain the inflammatory response in addition to its role in the formation of neuromuscular junctions during regeneration[81,117,178]. In axotomized sensory neurons, vasoactive intestinal peptide (VIP) and neuropeptide-Y are upregulated[208,228] while VIP and substance P increase in axotomized sympathetic neurons[63,228]. VIP may function by increasing blood supply to regenerating neurons[177] , and has been shown to enhance proliferation of astrocytes and release of a neuronal survival factor in the CNS[20]. CGRP is involved in sustaining the inflammatory response required for regeneration to occur. Both VIP and CGRP may also contribute to glial cell function by increasing cAMP levels which subsequently potentiate the effects of mitogenic growth factors on Schwann cells and endothelial cells of blood vessels[31]. These mitogenic factors include fibroblast growth factor (FGF), glial growth factor (GGF), and platelet derived growth factor (PDGF), and are released from transected axons, macrophages, platelets and activated Schwann cells[172,221].

Axotomized neurons also synthesize and release cytokines which contribute to the inflammatory response and synergize cytokines released by macrophages and other non-neuronal cells. Released cytokines include IL-1, IL-2, IL-6, TGF-β, and IFN-γ [100,141,174] (Fig. 1-1). These cytokines are mitogenic for

Schwann cells and influence their phenotype. TGF-β released from axotomized DRG neurons is mitogenic for Schwann cells and modulates the synthesis of NGF and deposition of ECM proteins by activated Schwann cells[174]. TGF-β is also involved in the stimulation of resident fibroblasts and subsequent collagen deposition, which can result in scar. This scar can result in a physical barrier to axonal outgrowth[5]. There are conflicting findings in the literature as to the significance of scar formation through TGF-β expression[4,5,43].

Other important sources of cytokines include macrophages and microglia. Following axotomy, macrophages and microglia proliferate, surround neuronal soma, release cytokines, express MHC I and II antigens and may participate directly in the cell body reaction[161,182]. Macrophages may contribute to altered neuropeptide expression in sympathetic ganglion cells via IL-1 induced release of leukemia inhibitory factor (LIF) from glial cells[167,182]. Expression of IL-6 and TGF-β from neurons, astroglia and/or microglia may contribute to expression of neurotrophic factors in astrocytes that surround axotomized motoneurons and DRG neurons via autocrine and paracrine pathways[63,100,141].

The Proximal Environment

Following axotomy, the axonal stump proximal to the site of transection degenerates back to at least the first node of Ranvier. Subsequent formation of growth cones occurs, with as many as 50-100 axon branches arising from the

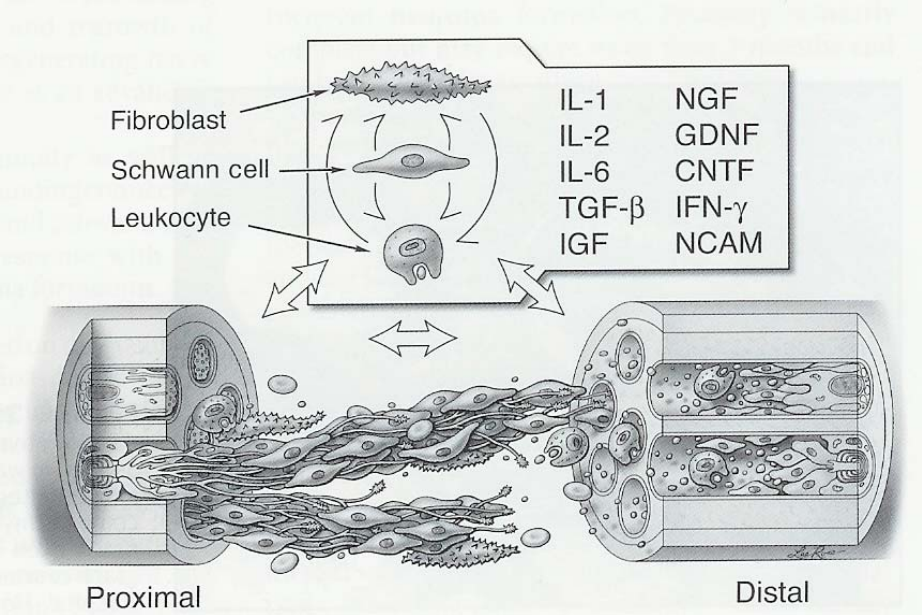

Figure 1-1: Growth factors involved in signaling inflammatory mediators and cytokines. These factors attract and activate Schwann cells and macrophages which are essential for nerve regeneration[118].

node of Ranvier. Growth cone formation occurs without direct support from the cell body and depends on neuronal elements locally available in the axon. Studies in vitro have shown that continued axonal transport, even in isolated axons, is sufficient to support growth cone formation[19]. However, materials provided by the cell body via axonal transport are the major source for subsequent axonal elongation, since regenerating axons have little capacity for independent protein synthesis during growth cone formation[42]. Axonal elongation is initially slow, but accelerates to reach a constant rate by the third day post-injury[80]. The rate of axon regeneration corresponds with the rate of slow transport that carries the cytoskeletal proteins essential for axon elongation[33,74,90,220]. The fate of growth cones is intrinsically dependant on the environment distal to nerve transection. With an unsupportive environment, growth cone branches continue to grow and spiral forming a neuroma[195,224]. However, with a supportive environment, growth cones grow toward the distal nerve stump and once within the distal stump, numerous fine nerve fibers grow out from the parent axons (Fig. 1-2). These axonal sprouts advance distally comprising a "regenerating unit"[138]. These units remain in the distal stump unless axons make target connections. Once this occurs, all but one regenerating unit are withdrawn, a process taking months to years [139]. Regenerating axons within the distal stump grow in diameter but do not approach normal size unless they make functional target connections[72]. The rate of axonal regeneration in humans ranges from 1-3mm/day following a 3-day latency period[74,90,91]. The ingrowth of growth cones into the distal nerve stump stimulates Schwann cell proliferation which in turn myelinates the newly formed

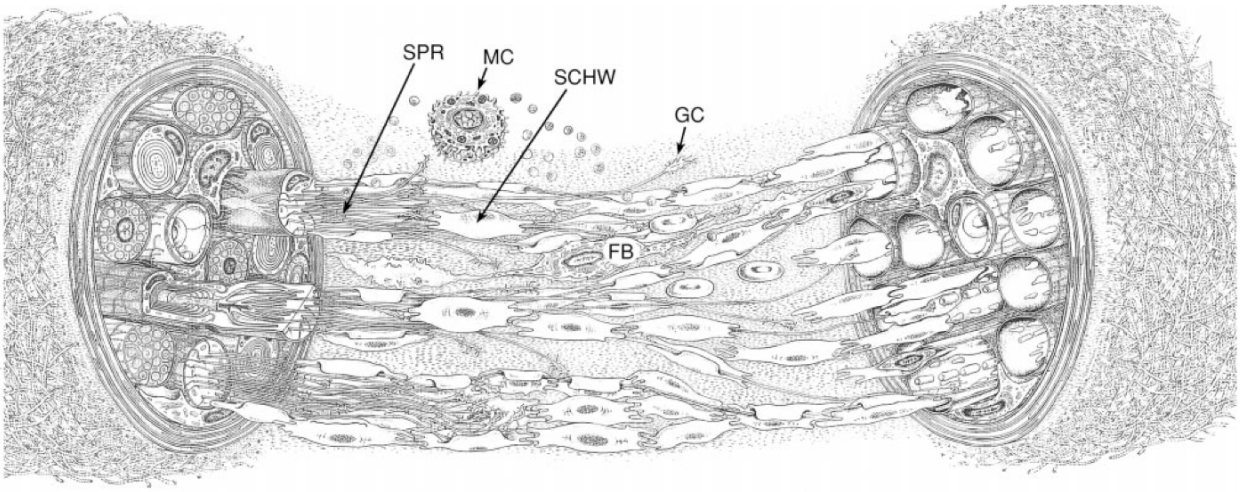

Figure 1-2: The local cellular response to nerve transection involves axonal sprouting at the transected proximal nerve segment. Sprouts (SPR) arising from one myelinated axon form a regenerating unit surrounded by a common basal lamina, at the tip of which is a growth cone (GC). Sprouts advance towards the distal nerve stump, through the zone of injury in immediate association with Schwann cells [219]. In the injury zone there are macrophages, fibroblasts (FB), mast cells (MC), and blood corpuscle elements. Sprouts attach to the bands of Büngner present in the distal nerve stump[118].

axons. Although Schwann cells synthesize the myelin sheath, it is the outgrowing axon size that determines the extent of myelination[87,187]. The initial myelination by Schwann cells within the distal nerve stump results in short inter-nodal distances. This limits conduction speeds in the regenerated nerves but gradually increases with rearrangement and increase of the inter-nodal distances[87,88].

The Distal Environment

The environment distal to the site of axotomy is essential for successful nerve regeneration. The permissive growth environment is a result of effective phagocytosis of myelin by invading macrophages and resident Schwann cells, and substrate support provided by non-neuronal cells including fibroblasts of the endo-, peri-, and epineurium as well as resident macrophages. Following injury, the distal stump undergoes a process of Wallerian degeneration whereby Schwann cells and macrophages clear the distal stump of axonal debris and myelin[160]. This is essential for regeneration as it opens the way for axonal in-growth and removes components of nerve and myelin sheath that directly inhibit axonal in-growth [160]. During the first 2 days after injury (when macrophage invasion is minimal), Schwann cells are the major participants in phagocytosis[170]. Within hours of injury, Schwann cells express MAC-2, a galactose-specific myelin which mediates non-immune opsonin-independent phagocytosis and two to three days following injury, macrophages invade the distal environment and express MAC-1, MAC-2 and Fc receptor[170]. The MAC-1 and Fc receptor mediate immune opsonin-dependent myelin phagocytosis[63,170]. In addition, macrophages release

growth factors and cytokines that stimulate Schwann cell and fibroblast proliferation, and stimulate the synthesis of growth factors and adhesion molecules by non-neuronal cells of the nerve sheath and endothelial cells of the blood vessels. In this capacity, macrophages play an integral role and are essential to successful nerve regeneration. Studies have demonstrated increased axonal outgrowth with application of exogenous macrophages[136,193]. Macrophages that have digested myelin secrete cAMP dependant mitogens including PDGF, FGF, and TGF-β (also released from platelets)[7,41,53,172]. TGF-β stimulates the proliferation of cells of mesenchymal origin but inhibits division of other cell types, particularly those of epithelial origin[174]. It also functions in inducing the pre-myelinating status of Schwann cells through down-regulation of p75 and regulation of myelin related proteins and type IV collagen[28,96,133,137,166]. Schwann cells, in addition to functioning in phagocytosis, proliferate in response to macrophage stimulation and absence of axonal contact[7]. As they multiply to fill endoneurial sheaths, they form longitudinal columns commonly known as bands of Büngner. In response in injury, Schwann cells not only proliferate, but transition from a state of myelination to non-myelination such that genes encoding myelin associated proteins are down-regulated, including P0, myelin basic protein (MBP), myelin associated glycoprotein (MAG) and peripheral myelin protein-22 (PMP-22)[44,111,202]. The non-myelination state is one of growth factor elaboration including neurotrophic nerve growth factor (NGF), neurotrophin 4/5, BDNF, endothelial growth factor (EGF), insulin-like growth factor (IGF) 1 and 2 and glia-dervied neurotrophic factor (GDNF) [63]. There is typically a second phase of

Schwann cell proliferation that occurs once regenerating axons enter the distal stump. This results in a 3-fold increase in the number of Schwann cells in the distal stump to form the myelin sheath of the newly entering axons[157].

Growth factors active in the distal environment include NGF, BDNF, and neurotrophin 3,4/5, which are important in preventing neuronal apoptosis in response to injury and potentiate the migration and adhesion of Schwann cells to axonal projections[18,65,134]. IL-6 also plays an important role by activating macrophages and other components of the immune response to enhance neuronal regeneration. IGF 1 and 2, FGF, PDGF, and GDNF all play a neurotrophic role in enhancing the environment in which regeneration takes place.

Cell adhesion molecules and basement membrane components are vital to neurotropism and are upregulated by Schwann cells to aid in contact guidance of growth cones[39,40,122]. Molecules including glycoprotein L1, neural cell adhesion molecule (N-CAM), N-cadherin, laminin and tenascin are required for adhesion of axons and Schwann cells, and for neuronal outgrowth within a conduit or acellular system[50,125].

The Effect of Fibrosis and Scar on Regeneration

The success of axon regeneration can be viewed as a balance between regeneration and scar formation. Fibroblasts are present in the epineurium and perineurium, and proliferate with injury. This is mainly due to the growth factor

TGF-β, secreted by activated Schwann cells and platelets following nerve injury[133]. TGF-β exists in 5 isoforms, the first three of which are relevant to neuronal function. TGF-β1 is generally expressed in response to injury and has multiple actions including chemotaxis of macrophages and fibroblasts. It stimulates the increased production of IL-1 by macrophages, enhances fibroblasts to produce extracellular matrix, collagen and fibronectin, and is a mitogen for Schwann cells. The effect of TGF-β on fibroblasts is of particular interest as histologically healing nerves demonstrate a large amount of fibrosis and scar, which could in fact be creating a physical barrier to axonal regeneration[4,5]. TGF-β2 and -β3 have recently been localized to neuronal tissues exclusively, both in the CNS and PNS of mice and rats[56,205]. TGF-β2 and -β3 have been shown to have a differentiative rather than mitogenic effect on neuronal tissue. In the CNS, TGF-β promotes astroglial migration and scar deposition. In the PNS, TGF-β modulates Schwann cell differentiation [206]. TGF-β has also been shown, both *in vivo* and *in vitro*, to act synergistically with neurotrophic factors in promoting neuronal survival: blockade of all three isoforms *in vivo* led to is a significant decrease in the survival-promoting effects of neurotrophic factors CTNF and FGF [107,108].

The adverse effect of scar and fibrosis on axon regeneration was demonstrated by Atkins *et. al.*[5] where an inverse relationship was found between scar tissue formation and axonal regeneration. Decreased scarring at repair sites showed improved compound action potentials and nerve conduction velocities.

Histological analysis revealed a decrease in nerve diameter and myelinated fiber count distal to nerve injury with increased scarring. A follow-up study by Atkins *et. al.* [4] investigated the effect of antibodies to TGF-β1 and TGF-β2 on nerve regeneration. A decrease in scar formation at the repair site was found but no improvement in compound action potential between the experimental and control groups could be demonstrated. These results were in contrast to an earlier study by Davison *et. al.* [43] which demonstrated improved compound muscle action potentials and whole muscle twitch strength in the rat gastrocnemius-soleus neuromuscular unit with the application of anti-TGF-β1 antibodies to the repair site. In addition, a study by Zou *et. al.* [229] demonstrated increased axonal regeneration and myelination in mice with the application of tissue plasminogen activator (tPA) to repair sites, providing further evidence for a potential role of scar inhibition in promoting axonal regeneration in the PNS.

A review of the literature reveals clear evidence to the relationship between scar tissue formation and axonal regeneration, though conflicting evidence remains pertaining to the effect of blocking the action of TGF-β in order to improve regeneration. Differences in results between Davison and Atkins could be attributed to the fact that Davison blocked only TGF-β1, while Atkin's protocol blocked both TGF-β1 and TGF-β2. It is known that TGF-β has mitogenic effects (particularly TGF-β2) on Schwann cells and promotes regeneration. Thus by blocking its action, a reduction in scar and fibrosis is achieved at the expense of regeneration.

Effects of Chronic Axotomy and Denervation

Chronic axotomy and denervation greatly limit functional reinnervation. Classically, poor functional recovery has been thought to be secondary to target muscle degeneration from long-term denervation, but new evidence using retrograde fluorescent labeling of neurons and cross-suture techniques have demonstrated that the effects of chronic axotomy and chronic denervation dictate regenerative capacity and limitations[61,62]. Chronic axotomy does not compromise the number of muscle fibers reinnervated following repair, however it does reduce the number of motoneurons able to regenerate their axons to muscle and thus is a contributing factor to poor functional recovery following nerve injury[61]. Chronic denervation results in a significant decrease in the ability of the distal nerve stump to support regeneration whereby many of the cellular changes present 1 week post injury decline after approximately 1 month and reach baseline levels at 6 months[62,194]. Endoneurial sheaths, once lined with active Schwann cells, macrophages and growth factors become atrophic[223]. In a study done by Boyd *et. al.*[18], application of the neurotrophic factors BDNF and GDNF to chronically axotomized neurons dramatically increased the number of regenerating motoneurons. The same application of neurotrophic factors to an acutely axotomized neuron failed to show any benefit. Thus, it would seem that growth factors secreted by denervated Schwann cells can enhance regeneration in chronically denervated neurons and by extension, in injuries where regeneration must take place over long distances. Further evidence presented by Sulaiman *et.*

al.[194] demonstrated the ability of chronically denervated muscle to survive and accept reinnervation. Thus, contrary to classic belief, it is the chronic denervation of Schwann cells and motoneuron units that limits functional recovery, not target muscle atrophy.

Neurotrophic Factors, Neurite Outgrowth and Axonal Growth

Neurotrophic factors and neurotrophism

Neurotrophic factors form the basis of neuronal survival and regeneration. Evidence suggests that they act directly to promote cell survival and indirectly (via non-neuronal cells) to promote regeneration. There are 3 major groups of neurotrophic factors: (1) neurotrophins, (2) neuropoietic cytokines and (3) fibroblast growth factors. In addition to these main groups, other growth factors include IGF, EGF, LIF and GDNF.

Neurotrophin Family

The neurotrophin family includes the following characterized growth factors: NGF, BDNF, neurotrophin 3 (NT-3), NT 4/5 and NT6. These factors bind to high affinity tyrosine kinase (trk) receptors and low affinity nerve growth factor receptor (p75).

NGF was initially described in 1953 by Levi-Montalcini *et. al.*[34] where the neurotrophic factor was shown to specifically enhance neuron survival and neurite outgrowth in sensory neurons. Further study by Arakawa *et. al.*[3] demonstrated little to no effect of NGF on motor neurons. NGF binds to trkA receptor which is present on sensory but not motor neurons. NGF is usually present in low concentrations in the healthy nerve, but upon nerve injury, its levels are greatly increased in the distal nerve segment of sensory nerves. NGF acts via the trk A receptor to enhance sensory nerve survival and regeneration. NGF is believed to exert its effect on motor neuron regeneration indirectly through non-neural cells. *In vivo*, exogenous NGF has no effect on regeneration of motor neurons but when applied to a silastic nerve bridge, it results in an increase in regeneration rate. This is thought to be secondary to an increased outgrowth of non-neuronal cells into the silastic chambers. Since migration of Schwann cells is an early prerequisite for regeneration across nerve bridges, NGF induced enhancement appears to be due to its indirect effect of promoting migration of non-neuronal cells [30]. Also, NGF increases angiogenesis, increasing blood flow to the site of regeneration thereby enhancing regeneration. Interestingly, Schwann cells do not express trk receptors, thus the actions of NGF might be mediated through the p75 receptor. There is also evidence that p75 may be involved in apoptosis and that binding of NGF to p75 prevents the apoptotic pathway[30].

BDNF is a trophic factor for motor neurons shown to support survival of motor neurons in culture [86], rescue developing motor neurons from natural cell

death and has a role in preventing cell death following axotomy [118]. BDNF acts through binding to trk b, trk c and p75 receptors found on motor neurons. A recent study demonstrated improved motoneuron regeneration rates and decreased neuropathic pain (as measured by autocannibolism) with exogenous application of BDNF following nerve transection injury [17]. A dose dependant relationship was found whereby low dose BDNF had beneficial effects on nerve regeneration while high doses of BDNF actually inhibited nerve regeneration. This is thought to be due to the action of BDNF on different receptors. The high dose inhibition of axonal regeneration was eliminated with the addition of blocking antibodies to the p75 receptor, indicating an inhibitory role for p75 in motoneuron regeneration [16].

NT-3 preferentially binds to trkC. This factor has been shown to strongly support motor neuron survival and growth in vitro [86]. NT – 4/5 binds to trk B and also supports survival of motor neurons. Studies have also demonstrated an increased ability of motor neurons to innervate skeletal muscle fibers when exposed to NT- 4/5 [222]. NT-6 is the newest member of this group. Its effect on motor neurons is currently unclear [118].

Neuropoietic cytokine family

The neuropoietic cytokine family includes ciliary neurotrophic factor (CNTF), IL-6, oncostatin M, granulocyte colony stimulating factor (GCSF) and LIF. Members of this family share common structural motifs, bind to common receptor components and use the JAK/STAT signaling pathways. Expression of

these molecules after nerve injury suggests an involvement in promoting the survival of injured neurons in both the central and peripheral nervous systems.

CNTF facilitates the survival of dorsal root and autonomic ganglion neurons, motoneurons, and hippocampal neurons. *In vivo*, CNTF rescues chick embryonic motoneurons from naturally occurring cell death [150]. It also prevents early death of motoneurons in a murine model of motoneuron disease [63]. As shown for neurotrophins, deletion of CNTF receptor is more deleterious than deletion of the CNTF gene itself, as demonstrated by extensive motoneuron death in the CNTF receptor knockout mouse[45]. Schwann cell damage due to nerve injury may trigger release of CNTF[191]. The availability of CNTF is limited because of its down-regulation in the distal nerve stump within the first day of injury and thus CNTF is less effective in preventing axotomy-induced motoneuron death than neurotrophins[60,183]. CNTF has been shown to play an active role in regeneration. Systemic or local infusion of CNTF increased the rate of axonal elongation, number of reinnervated muscle end plates, muscle mass and functional recovery[146,176,204].

LIF and IL-6 are both upregulated in the distal stump after nerve injury. LIF has been shown to promote survival of embryonic motor and sensory neurons *in vitro*. IL-6 is a stress related protein which is strongly induced after tissue injury[37,184]. IL-6 is produced by fibroblasts, macrophages, mast cells, and Schwann cells which are stimulated by IL-1, TNF-α, LIF, oncostatin M and IL-

6[15,23,147,209]. IL-6 is induced within hours of injury and remains elevated for 3 weeks. This coincides with elevated numbers of macrophages and fibroblasts, which are the primary producers of IL-6. Studies to this effect have shown decreased levels of IL-6 in mice with deficient macrophage recruitment[169]. *In vitro*, IL-1 and TNF-α have both been shown to induce IL-6 production, although *in vivo*, TNF-α is expressed too late to induce IL-6 after axonal injury [192]. The role of IL-6 in promoting regeneration is unknown. It may play a role in promoting survival of axotomized neurons and neuronal response to injury. The early upregulation of IL-6 in the distal nerve stump following injury, mainly by fibroblasts, suggests a potentially important role in promoting the infiltration of blood-borne cells into the degenerating nerve stump. These cells include macrophages and lymphocytes, which in turn, release cytokines (IL-1, IL-6, TNF-α, and IFN-γ) that increase IL-6 production. These cytokines also induce the expression of MHC I and II on Schwann cells suggesting a potential role in T-cell mediated immunocompetence and rejection[102,215].

FGF is another growth factor shown to promote axonal outgrowth in developing neurons despite decreased levels post-injury[114]. It does this by enhancing regeneration of axons across collagen filled nerve stumps[1]. FGF also has a mitogenic effect on Schwann cells and promotes angiogenesis[68].

Other growth factors implicated in nerve regeneration

Other growth factors including insulin like growth factor (IGF) I and II, GDNF, Glial maturation factor β (GMF-β) and PDGF have also been shown to contribute to regeneration.

IGF-I and II are upregulated in the distal stump following axotomy and down regulated once regenerating axons reach the distal stump implying a neurotropic role in guiding regenerating axons from the proximal to distal stump. Low concentrations of IGF *in vitro* enhance the survival of sensory, sympathetic, and motoneurons [63]. *In vivo*, exogenous application of IGF-I rescues lumbar motoneurons from both naturally occurring and injury-induced neuronal death in chick embryos [144]. In neonatal mice, IGF-I completely prevents motoneurons from axotomy induced neuronal death [113]. IGF-II also has survival promoting effects on lumbar motoneurons in chick embryos [144]. In terms of axon regeneration, both IGF-I and II promote neurite outgrowth of embryonic sensory, motor and sympathetic neurons *in vivo* when delivered locally after crush, freezing and transection injuries of the sciatic nerve [189]. Also, injured nerves treated with antibodies to IGFs show decreased axon regeneration. IGFs have both direct and indirect effects on regeneration. *In vitro,* IGF can effect increased neurite growth without the presence of Schwann cells[168]. *In vivo*, IGF –I promotes proliferation of Schwann cells and their migration into acellular nerve grafts[188].

GDNF is a distant member of the TGF-β family. It is expressed in the rat limb bud, embryonic myotubules, and Schwann cells [86]. RNA transcripts for GDNF have been found in Schwann cell lines both *in vitro* and *in vivo* [203]. *In vivo*, GDNF has been shown to be a potent survival promoting factor for embryonic spinal motoneurons and adult motoneurons [86]. GDNF is transported via retrograde transport to the cell body of neonatal facial and spinal motoneurons and completely prevents axotomy induced neuronal death and atrophy [149]. It has also been shown to be upregulated in the distal nerve stump following injury suggesting a role in axon regeneration. The application of exogenous recombinant GDNF has marked stimulatory effects of neurite outgrowth of sympathetic and ciliary ganglion neurons *in vitro* [52]. *In vivo* application of GDNF has shown an increase in axonal regeneration in models of chronic axotomy; no benefit for GDNF application has been demonstrated for acutely repaired nerves [17]. Unlike BDNF, GDNF does not have an inhibitory or dose dependant effect on motoneuron regeneration. Interestingly, a study by Boyd and Gordon [17] demonstrated a synergistic effect on motoneuron regeneration resulting from the exogenous application of BDNF and GDNF. This was explained by both factors acting on synergistic pathways.

Glial maturation factor β (GMF-β) is synthesized in Schwann cells in the distal nerve stump following injury. Its mechanism of action has yet to be elucidated.

PDGF, released primarily from platelets following injury, promotes neurite outgrowth and stimulates Schwann cell proliferation. PDGF exerts its main mitogenic effects on Schwann cells and endothelial cells, promoting angiogenesis.

Other factors with mitogenic effects include TGF-β, FGF, and EGF. TGF-β and PDGF released from damaged axons and infiltrating macrophages help to recruit macrophages to the injury site and stimulate synthesis of other growth factors such as FGF, EGF and IL-1 by macrophages [63]. In effect, TGF-β and PDGF amplify and prolong the duration of macrophage invasion, activation and synthesis. Consequently, a growth supportive environment is induced by promoting Schwann cell proliferation and expression of their "growth supportive" state.

Neurotropism

Several factors influence the direction of growth of regenerating axons. The concept of neurotropism was fist described by Cajal in 1928, where it was noted that the axons of a transected nerve grow preferentially toward the distal nerve stump. It was hypothesized that this phenomenon is mediated through diffusible factors attracting the advancing growth cones. This was demonstrated *in vivo* by Kuffler *et. al.* [109] where growth cones preferentially advanced toward distal nerve stumps in the frog. In the absence of a distal nerve stump, the

advancing growth cones grew randomly. The concept of regenerative specificity is complex and multifaceted. Regenerative specificity can be described as tissue specificity (guidance of regenerating axons to distal nerve stump instead of other tissues like muscle or tendon), fascicular specificity (re-alignment of originally attached fascicles), sensory vs. motor specificity, topographic specificity (return of axons to the area they previously served, such as middle finger axons to middle finger following median nerve repair) and end organ specificity (re-innervation of end organ by axon that originally innervated it) [118].

Cell Adhesion Molecules (CAM)

An essential part of neuro-regeneration is neurotropism, that is, the ability of regenerating neurons to be guided to the appropriate targets. Cell adhesion molecules play a vital role in neurotropism. Members of this family of proteins include L1, Ng-CAM, NILE, N-CAM, N-Cadherin, and extracellular matrix proteins (ECM) such as laminin, fibronectin and tenascin-C.

L1 and N-CAM are cell adhesion molecules expressed only by non-myelinating Schwann cells. MAG is a cell membrane glycoprotein believed to be involved in myelination during regeneration and expressed only by myelinating Schwann cells[125]. During or after axonal degeneration, MAG is down regulated and Schwann cells form slender processes that are L1 and N-CAM positive[124]. These two molecules have been shown to be involved in axon-to-Schwann cell and axon-to-axon adherence. They bind to like molecules in a calcium

independent manor. They also mediate cell adhesion by binding to other CAMs and to integrins[12]. Integrins belong to the family of α-β heterodimeric receptors and transmembrane proteins. They interact internally with the cytoskeleton and externally with ECM molecules. L1 and N-CAM may synergize with neurotrophic factors such as survival-promoting molecules for axotomized neurons, particularly as integrins and neurotrophins converge on the same components of the signaling pathway. Functional capacities of L1 and N-CAM can be modulated by binding to carbohydrate moieties such as L2, HNK-1 and polysialic acid (PSA). L2 and HNK-1are carbohydrate epitopes known to be associated with cell adhesion molecules [110]. L2 and HNK-1 are expressed by L1, N-CAM, MAG, and tenascin whereas PSA is restricted to N-CAM[180]. Preferential expression of L2 and HNK-1 on Schwann cells in the motor pathway after nerve injury has been linked to significant preference of regenerating motor axons for motor rather than sensory nerve branches in young animals *in vivo* [118]. PSA is expressed in a unique subset of motoneurons and is dramatically upregulated with axotomy. Recently, PSA was shown to play a role in motor axon guidance during regeneration where select motoneuron pools expressing PSA were shown to preferentially reinnervate muscle[58]. Additionally, further stimulation of PSA expression with electrical stimulation resulted in a larger number of motoneurons regenerating their axons to correct muscle targets.

N-cadherin is normally expressed on the Schwann cell membrane where it comes into contact with other Schwann cells[185]. It has also been shown to be

expressed in the distal nerve stump following injury[32]. When axons regenerate into the distal nerve stump, N-cadherin immunoreactivity is localized to the axon and Schwann cell membrane where axon-axon or axon-Schwann cell contact is made. This suggests the N-cadherin might be involved in axonal growth by mediating adhesion between axons and Schwann cells[185]. N-cadherin either as a substrate or transfected into heterologous non-neuronal cells has potent effects in stimulating neurite outgrowth *in vitro*[13]. This effect is partially blocked with application of antibodies to N-cadherin [63].

ECM proteins are found on the basal lamina and endoneurium. They are synthesized by Schwann cells and fibroblasts residing in the peripheral nerve. They include laminin, fibronectin, entactin/J1/tenascin, heparin-sulfate-proteoglycan (HSPG), and type 4 and 5 collagen. Axonal contact is required for elaboration of the basal lamina by Schwann cells[24]. Nevertheless, some components of the lamina (e.g. Laminin) continue to be synthesized under denervated conditions. The lamina remains intact for 3 to 4 weeks post nerve injury and then becomes fragmented. This fragmentation of the lamina over time likely contributes to poor axonal regeneration and target reinnervation after delayed nerve repair[62]. *In vivo* studies have demonstrated the essential role of laminin (and possibly tenascin) in providing substrate for supporting axonal regeneration. In addition to providing substrate for growth, ECM molecules bind to and regulate the activity and stability of several growth factors, most notably FGF and TGF-β [63].

Laminin is present in nervous systems that are able to regenerate and thus is absent in the human CNS. In the PNS, regenerating axons fail to cross silicone or collagen grafts unless laminin is present. The presence of Schwann cells and laminin are necessary for successful regeneration. A study by Wang *et. al.* [214] demonstrated the importance of laminin to early axonal regeneration showing a marked decrease in axonal regeneration through nerve grafts treated with anti-laminin antibody. Laminin was also found to have a positive effect on perineural cells including Schwann cells. Further evidence of the role of laminin in axonal regeneration was presented by Tong *et. al.* [201] where artificial nerve conduits lined with laminin resulted in greater regeneration than control conduits devoid of laminin.

Tenascin C is normally confined to the ECM around the node of Ranvier and the perineurium[126]. It is expressed along the entire length of the distal stump and distal end of the proximal stump following peripheral nerve injury and forms a bridge between the two stumps. The spatial and temporal expression of tenascin and its growth promoting effects *in vitro* suggest a possible involvement in axonal regeneration[126]. It is probable that IL-1 and TGF-β play a role in the upregulation of tenascin in Schwann cells[130,156]. Sustained release of tenascin in chronically denervated nerve stumps and down regulation by regenerating axons suggest that loss of axonal contact is a sufficient signal to induce tenascin expression[126].

The Clinical Problem

Although nerve regeneration has been shown to occur in the PNS, functional outcomes are poor, especially in proximal injuries resulting in a long regenerative distance. As discussed earlier, the problem lies in the time for regeneration to take place and the ability of the distal environment to maintain Schwann cells and macrophages in a state of growth factor elaboration conducive to regeneration. For example, brachial plexus injuries result in denervation of distal nerve pathways and a regeneration distance of over 800mm. In a system where the environment distal to injury remains conducive to regeneration for approximately 1 month, it is not surprising that functional outcomes of nerve repairs are poor [63,73]. In addition, studies examining regeneration through nerve repair sites document that the limiting step is traversing repair coaptation sites – single in primary repairs and double in graft repairs. Thus there are two main hindrances to nerve regeneration in the PNS – traversing coaptation sites and maintaining an environment supportive to regeneration, including expression of structural, neurotropic and neurotrophic factors.

Current Strategies in Nerve Repair

Understanding the obstacles to regeneration forms the basis of current research to enhance nerve regeneration in the PNS. Strategies to enhance peripheral regeneration can be divided into 2 categories: technical and biological.

Technical advancements in nerve repair

The ideal repair for a peripheral nerve defect is primary coaptation of the two severed nerve ends (Fig. 1-3 and 1-4) [118]. Segmental defects where a tension-free primary coaptation cannot be performed present a challenging reconstructive problem. In such cases, a conduit has to be introduced to bridge the defect. Using this method of repair requires regenerating neurons to traverse two suture lines and a conduit which is conducive to regeneration. Autologous nerve grafts remain the mainstay of treatment, but it requires the sacrifice of a normal healthy nerve. Thus, alternate conduit materials are actively being investigated.

Autologous Nerve Graft

Autologous nerve grafting has evolved into the current gold standard in peripheral nerve repair. The purpose of using an autologous nerve graft is to provide a conduit consisting of a basal lamina together with their corresponding Schwann cells [118]. Studies have shown than conduits containing Schwann cells support axonal regeneration more efficiently than grafts composed of basal lamina alone[76]. This is likely attributed to the ability of Schwann cells to synthesize

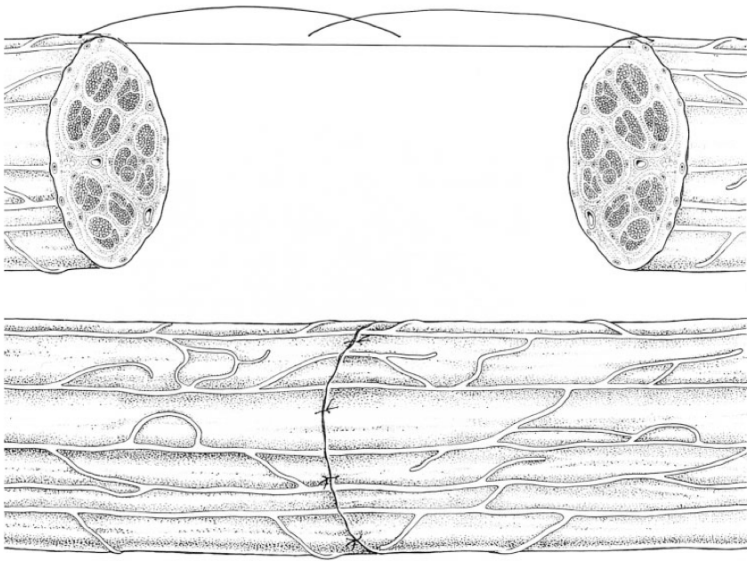

Figure 1-3: Epineural suture. Coaptation is achieved through suturing the epineurium of the proximal and distal ends of the transected nerve. Proper alignment can be achieved by using blood vessels as surface landmarks, however, fascicular bundles will not necessarily be in proper alignment[118].

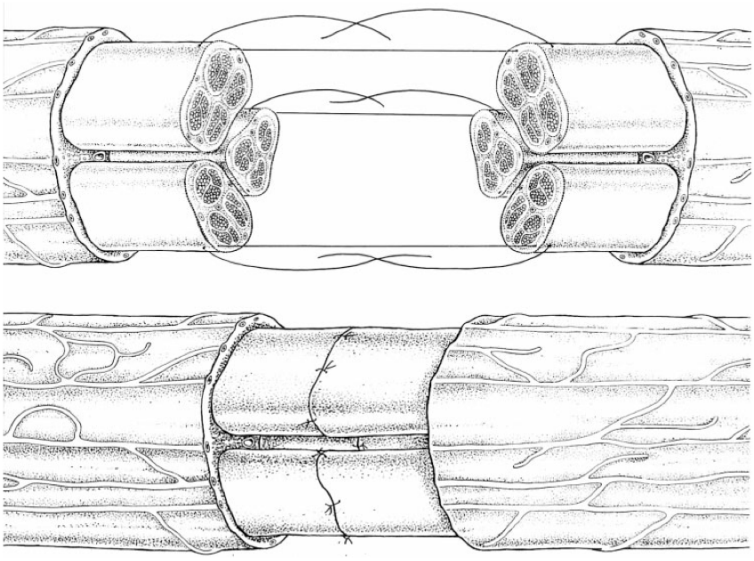

Figure 1-4: Fascicular suture. Epineural tissue is resected and individual fascicles are coapted with sutures in the perineurium [118].

neurotrophic factors and extra-cellular matrix components including laminin and fibronectin. In addition to the Schwann cells, the presence of laminin and fibronectin in the basal lamina of the graft is essential to regeneration, where antibodies to fibronectin and laminin inhibited axonal regeneration through sciatic nerve grafts [214]. Various types of experimental manipulation of autologous nerve grafts, such as electrical stimulation, vibration exposure and hyperbaric oxygen, have shown improved regeneration [118]. These manipulations have not yet transcended to clinical application.

Allograft

Allografts are nerve grafts harvested from a genetically mismatched donor and are an attractive alternative to autologous nerve grafts as they eliminate donor site morbidity. The main factor limiting the use of these grafts is rejection. Studies have shown that in time, the donor Schwann cells present in the allograft are replaced with host Schwann cells, thus recent efforts have focused on short term immunosuppressive regimes to promote immunotolerance until the host Schwann cells have infiltrated the graft. Once replaced by host cells, the graft has been shown to function as an autologous graft, without further need for immunosuppressive agents.

Immunosuppression for allograft tolerance is a current area of active research. Nakao and Mackinnon [142] demonstrated successful immunotolerance and regeneration through an allograft using monoclonal antibodies to the surface receptors ICAM-1 and LFA-1. Another approach to induce immunotolerance is

making the allograft acellular. Gulati *et. al.* [77,78] demonstrated successful immunotolerance and regeneration through acellular grafts in rat and rabbit models, though regeneration was not as robust as that through a cellular autograft. The limiting factor in acellular grafts would seem to be the lack to growth factor producing Schwann cells. Improvement in regeneration through acellular allografts has been demonstrated with the addition of VEGF [190] and bFGF[94]. Additionally, cultured autologous Schwann cells have been used in these grafts with promising results [51].

Clinical application of allografts is limited, mainly due to rejection. One clinical case study presented by MacKinnon [120] showed minimal sensory and no motor recovery following the use the 8 allografts for the reconstruction of an extensive tibial nerve defect (20 cm gap) in a 12 year old boy. As immunosuppressive regimes improve, the use of allografts will no doubt become more prevalent as it circumvents one of the major consequences of autologous nerve grafting, donor site morbidity.

Muscle Grafts

Nerve regeneration is dependant upon an adequate basal lamina and thus any conduit providing such a lamina is potentially a suitable candidate for a nerve graft. One such candidate is acellular muscle grafts. These conduits provide an adequate basal lamina containing both laminin and fibronectin. Muscle grafts are rendered acellular either by a freeze-thawing procedure or by other chemical

methods [69]. Migration of Schwann cells into these grafts is essential for regeneration to occur, and thus these grafts are length limited [54]. The length limitation of these muscle grafts is discussed by Hems et. al. [85] where a study comparing 5cm and 10cm muscle grafts was performed. The 10cm muscle grafts failed to support adequate nerve regeneration when compared to the 5 cm muscle graft as well as the 10cm control nerve autograft. Studies using interposing nerve segments within the graft to provide an additional Schwann cell source have improved the regenerative capacity of these grafts. Clinically, these grafts are appealing as there is an unlimited source of muscle tissue that can be harvested with no resulting morbidity.

Other Types of Conduits

Materials other than nerve tissue have also been studied, all in an attempt to maximize nerve regeneration while minimizing donor site morbidity. In addition to muscle grafts, venous grafts have been used in experimental models with promising results [57]. Biosynthetic conduits are a novel area of research. Various conduits made of different materials have been and are currently being investigated including silicone, polyglactic acid and collagen. Successful regeneration has been demonstrated using silicone conduits, although the length of graft is limited due to the fact that silicone is impermeable and thus does not allow the diffusion of growth factors. Newer bio-resorbable conduits such as collagen based nerve guides have shown encouraging results in rodents and primates. Laminin and fibronectin coated biodegradable collagen grafts have

been used successfully in rats [201]. Artificial nerve guides provide an attractive alternative to autologous tissue as they do not require the sacrifice of healthy tissue. Schwann cell invasion is critical for regeneration to occur. Once a defect is bridged by an artificial nerve guide, a fibrin-fibronectin cable forms between nerve ends through the guide and forms a substrate for both the migration of endothelial cells for neovascularization and Schwann cells, which in turn form the substrate for regenerating axons. This fibrin-fibronectin bridge is only effective for bridging short distances, and herein lies the limitation of clinical application of these nerve conduits. Acceptable regeneration through artificial conduits has been limited to 30mm, but a more recent study by Suzuki et. al.[196] demonstrated regeneration across a 50 mm sciatic nerve defect using polyglycolic mesh and alginate gel. Artificial nerve guides confer many advantages to nerve regeneration including allowing for the accumulation of neurotrophic factors and formation of fibrin clot, which allows for longitudinal advancement of axons from proximal to distal stump. Their use also minimizes surgical trauma to nerve ends and their application is technically simple.

Alternative Surgical Repair Techniques

There are situations where traditional end-to-end nerve grafting is unavailable or insufficient such as in proximal nerve injuries with long regenerative lengths, injuries where the proximal stump of the transected nerve is unavailable for coaptation, and injuries resulting in long nerve gaps. In brachial plexus injuries, for example, the current accepted method of treatment is cable

grafting, but with such a proximal nerve injury, the time to regenerate to the end target muscle is often too long to achieve functional reinnervation[79]. It has been shown that axonal regeneration through nerve stumps that have been chronically denervated for 12 weeks approaches only 20% of normal[194] and thus alternative or additional supportive measures are required for proximal nerve injuries whereby the distance to the end muscle requires a prolonged time for regeneration to occur.

To overcome these problems, the concept of alternative surgical repair methods has emerged consisting of end-to-side and side-to-side nerve repairs. Viterbo *et. al.* revisited the end-to-side technique in 1992, which consists of taking the distal stump of a transected nerve and inserting it into a pre-formed epineural window of a donor nerve [210-212]. In their studies, they demonstrated both histologic and electrophysiologic reinnervation using end-to-side coaptation. A number of subsequent studies have been published in both experimental models and clinical cases studies, however the data for end-to-side nerve bridges to date show conflicting results [163,179]. Complicating the issue is the question as to whether true collateral sprouting occurs from nodes of Ranvier or whether donor axonal injury is required for axons to regenerate through the side-to-side bridge[21,83,155]. Regardless of the mechanism, regenerating axons from the donor nerves are essential for the success of this technique. From the literature, sprouting occurs more frequently in sensory axons as compared to motor axons[127,197].

In the human PNS, there exists supportive and redundant lateral connections between nerves, most notably in the upper limb between the ulnar and median nerves, namely Martin-Gruber and Riche-Cannieu anastomoses. Based on these lateral topographic arrangements in the PNS, the side-to-side nerve repair technique was first described by Yuksel *et. al.* whereby epineural windows were created on the sides of a donor and recipient nerve and then coapted together in a lateral fashion. The study described a direct comparison between end-to-end, end-to-side and side-to-side nerve repairs[225]. Histological analysis and functional outcome measures indicated that the end-to-end repair resulted in the best regeneration followed by side-to-side nerve repair. End-to-side nerve repair yielded the least functional and histological regeneration. In 2004, a case report was presented whereby a high ulnar nerve laceration was repaired via end-to-end coaptation and supported distally by a side-to-side nerve bridge between the median and ulnar nerves[226]. Clinical follow-up revealed return of protective sensation as a result of the distal side-to-side nerve coaptation. Despite the promising results of these studies, there has been no further investigation in the current scientific literature evaluating the use of side-to-side nerve coaptation in injuries of the PNS

Biological Approach to Nerve Repair

Recently, the focus of nerve regeneration has shifted from technical to biologic modification. As understanding of nerve physiology and repair improves, biological factors are being investigated for potential beneficial effects

on nerve regeneration. Unlike technical advancements in nerve injury which have focused primarily on nerve regeneration, the biological approach to nerve injury focuses on two main processes: neuronal cell survival following injury and subsequent regeneration of surviving neurons.

The Use of Neurotrophic Factors

Neurotrophic factors, as discussed earlier, are natural substances expressed by neuronal and peri-neural cells during regeneration. Knowledge of these factors has led to investigation of the possible benefit of exogenous application of these growth factors on nerve survival and subsequent regeneration following injury.

Following transection injury, dorsal root ganglion cells are susceptible to apoptotic cell death. Application of NGF to repair sites has resulted in a significant improvement in dorsal ganglion cell survival [173]. In these studies, NGF-impregnated fibronectin bridges where used to fill nerve gaps. Similar effects have also been shown using NT 3 and IL-1 [118]. The potential clinical applications of neurotrophic factor support of cell survival holds much promise, especially in diseases where neuronal cell death is the primary pathophysiology such as central neurodegenerative diseases. Diseases like Parkinson's and Alzheimer's disease could potentially benefit from such an effect. In addition, traumatic peripheral nerve injuries in which an element of neuronal cell death has been implicated in poor functional outcomes also stand to benefit from such therapies.

In addition to supporting neuronal cell survival, exogenous application of NGF can improve regeneration when incorporated into an artificial nerve conduit or applied directly to a primary repair [217]. Other neurotrophic factors have also been shown to enhance neuronal regeneration. In a study by Lewin *et. al.*[112], the local application of BDNF and CNTF resulted in improved rate and degree of functional recovery in a rat sciatic nerve following transection injury and primary repair. In contrast, work by Gordon and Boyd[16] demonstrated that administration of BDNF to an immediately repaired nerve resulted in no improvement in the number of motoneurons regenerating axons across the repair site. However, administration of BDNF at low doses did significantly increase the number of motoneurons regenerating axons in a chronically axotomized nerve[16]. Both local and systemic administration of CNTF have increased the number of myelinated axons distal to the nerve repair site 4-6 weeks following injury [176]. Extrinsic IGF-1 applied locally to sciatic nerve injury has also shown an increase in axonal regeneration speed in a concentration dependant manor [188]. Treatment with antibodies to IGF-1 results in inhibition sensory axon regeneration. FGF has also been shown to promote axonal regeneration in experimental models [64].

Although numerous experimental models, both *in vitro* and *in vivo*, have demonstrated improved neuronal cell survival and regeneration with the exogenous application of neurotrophic factors, no clinical applications have been attempted to date. Many questions remain before these factors can be applied

clinically including toxicity, dosages, mode of administration and potential carcinogenicity.

Other Factors to Promote Nerve Regeneration

In addition to neurotrophic factors, other factors have been investigated as potential promoters of neuronal regeneration including gangliosides, various hormones, and electromagnetic fields. A newer agent FK506, also known as tacrolimus, has been recently shown to improve axonal outgrowth in experimental models [71]. This agent is currently used clinically as an immunosuppressant in transplant patients. The effects of FK506 are two fold: immunosuppression and promotion of axonal regrowth. In light of these two characteristics, its potential application to allografting holds promise and requires further study.

During nerve injury, clearance of myelin and other inhibitory glycosaminoglycans (GAG) in the distal nerve stump by Schwann cells and macrophages is essential for successful in growth of regenerating axons. The prolonged time course for phagocytosis of myelin[6,55] and degradation of GAGs[92] may account for staggered outgrowth of axons from the proximal nerve stump[2,73]. Overcoming these inhibitory mechanisms has been the focus of two experimental agents: rolipram and chondroitinase ABC. Rolipram functions to increase cAMP levels in the neuronal cell body. Injured neurons in the CNS can be stimulated to regenerate their axons despite the presence of inhibitory myelin associated molecules in the environment[26,145,165] by up-regulating neuronal cAMP. The

effect of elevating neuronal cAMP in the PNS is controversial in the literature with some studies demonstrating increased axonal outgrowth[67,101,162] while other studies have failed to demonstrate an effect on axonal outgrowth[14,82,131]. Degrading inhibitory GAGs at the site of nerve injury using chondroitinase ABC has been shown to promote axonal outgrowth into distal nerve stumps and acellular nerve grafts in the PNS[106,230]. Although acting on different targets, both strategies are aimed at overcoming inhibitory elements in the environment. Combination therapy using these two agents has yet to be investigated in the PNS.

An environmental factor that has been suggested to promote nerve regeneration is electromagnetic field application. A recent study by Longo *et. al.* [116] demonstrated that pulsed electromagnetic fields influence NGF activity and levels following sciatic nerve transection injury in rats. Hyperbaric oxygen treatments have also been investigated in animal models with mixed results. Use of neither technique has been reported in the clinical setting to date.

Exogenous Schwann Cells in Nerve Regeneration

Schwann cells are vital for structural myelination as well as growth factor secretion. By increasing the number of Schwann cells at the site of injury, one might increase the supply of growth factors and structural support proteins required for nerve regeneration for a longer period of time. Schlosshauer *et. al.*

[181] examined the effect of Schwann cells on axonal regeneration and found a significant enhancement of regeneration *in vitro*. More recent studies have demonstrated enhanced *in vivo* regeneration through nerve conduits with incorporation of Schwann cells [22,98]. Both histological and functional regeneration have been achieved. Although experimental models support the incorporation of Schwann cells into peripheral nerve repair models, there exist limitations to their use. The main limitation to Schwann cell use is that they must be harvested from donor nerve and thus a healthy donor nerve must be sacrificed leading to donor site morbidity. This presents the same problem as using autografting, the gold standard. An additional obstacle is that Schwann cell culture expansion is directly related to patient age, so this therapy would only be suitable for younger patients. The recognition of the beneficial effect of Schwann cells was vital however in searching for other cell types that may provide similar trophic and tropic support, namely MSCs.

Mesenchymal Stem Cells

Stem cell therapy is a rapidly expanding field with much promise in selectively enhancing regeneration and repair of tissues damaged by disease or traumatic processes[104]. Stem cells are defined as cells with self-renewing capability and the ability to differentiate into multiple cell lineages. Stem cells are generally divided into two main groups: embryonic stem cells and adult stem cells. Embryonic stem cells are technically classified as totipotent stem cells –

cells which retain the ability to form a whole organism. Once an embryo reaches the blastocyst stage of development, cells have specialized to pluripotent stem cells – cells with the ability to form any tissue in the body but not able to reform a whole organism. Adult stem cells are multipotent in that they retain the ability to differentiate into multiple, but not all, cell lines (Fig. 1-5).

The role of adult stem cells is to maintain and replace terminally differentiated cells that have expired secondary to injury or cell turn over. They have classically been described as tissue specific in that they reside in the mature tissue that they serve to maintain, implying that they are differentiated to the point of tissue specificity. Recent research has challenged this paradigm and suggested that tissue specific stem cells retain the ability to cross-over and differentiate into other cell lines – a concept that has been coined developmental plasticity.

Hematopoietic tissue harbors many stem cell populations including hematopoietic, mesenchymal and epithelial stem cells, as well as multipotent adult progenitor cells and endothelial precursor cells [104]. Recently, the scope of stromal stem cell plasticity has extended into neuronal tissue with numerous studies showing differentiation of MSCs into neuronal phenotypes *in vivo* – both in the central and PNS. For many years now, MSCs have been used as a natural source of neurons and glial cells that have been implanted into damaged central nervous system tissue to enhance tissue regeneration and function [35]. They have

been studied in mouse models for the treatment of Parkinson's disease, spinal cord injury and traumatic brain injury.

Identification of Mesenchymal Stem Cells

By definition, stem cells are identified as cells able to proliferate without differentiating while retaining the ability to differentiate into multiple cell lines. At present, the existence and characterization of MSCs is poorly defined. Most of the information currently available on MSCs is derived from indirect evidence from *in vitro* culture systems of bone marrow and other tissues. To date, no specific markers have been defined for the mesenchymal stem cell and little is known about its actual *in vivo* location, engraftment and behavior. Evidence from current in vitro studies must be interpreted with caution, as by nature, *in vitro* culture systems add an element of artifact. Such was demonstrated in studies done by Rombouts *et. al.* [175] who compared the homing abilities of primary and culture – expanded MSCs in a mouse model. The uncultured MSCs demonstrated homing to bone marrow but the infusion of culture-expanded MSCs failed to show lympho-hematopoietic localization.

Thus far, the identification of mesenchymal stem cell lines has depended on *in vitro* culture lines resulting from the ability of these stem cells to adhere to plastic surfaces and demonstrate multiple cell line differentiation. The first direct evidence of the existence of MSCs in the bone marrow came from experiments done by Friedenstein *et. al.* [59] which showed the presence of an adherent fraction

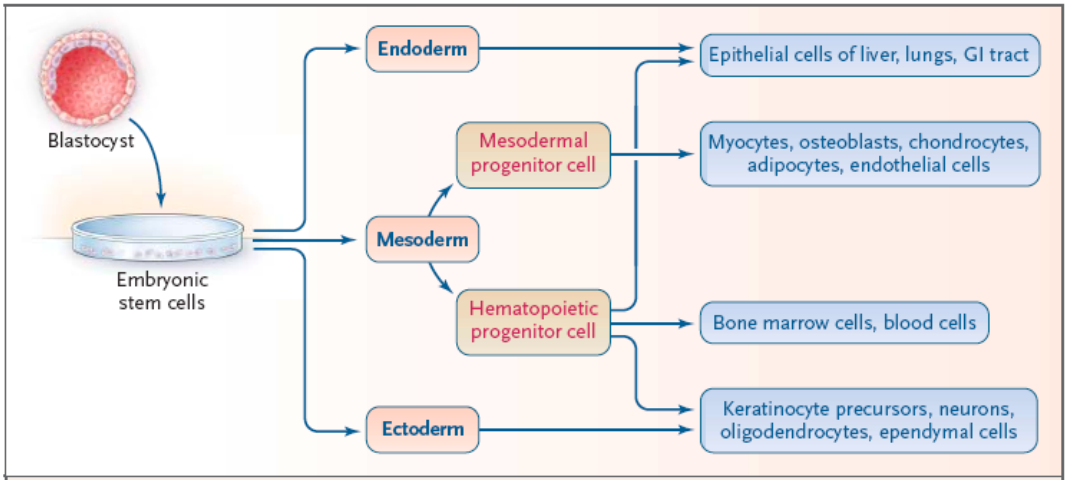

Figure 1-5: Embryonic stem cell lineages. Mesenchymal stem cells are derived from mesodermal lineage of embryonic stem cells and retain a high degree of pluripotency. They are capable of differentiation along multiple mesodermal cell lineages and more recently have been shown to differentiate along neuronal cell lineages[104].

of fibroblast like cells from the bone marrow which could differentiate into cells that could from small deposits of bone or cartilage. These cells were labeled colony forming unit – fibroblasts (CFU-F). Further studies demonstrated the multipotency of CFU-Fs, with differentiation into osteoblasts, chondroblasts, adipocytes, and myoblasts [164]. The frequency of CFU-F in the bone marrow is species dependent and is influenced by culture conditions [186]. Growth factors stimulating CFU-F proliferation include platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, transforming growth factor beta, and insulin-like growth factor. Factors inhibiting proliferation include IL-4 and IFN-α [10]. The formation of CFU-F has been considered indicative of MSCs but a direct relationship between the two has not clearly been established due to great variability in morphology, size and differentiation potential observed between species and individual colonies[10].

Marrow stromal cells are all cells within the bone marrow that are not part of the hematopoietic system. The stromal cell compartment consists of macrophages, fibroblasts, adipocytes and endothelial cells. These cells function to support hematopoietic cells. Stromal cells produce ECM components and both soluble and membrane-associated growth factors to form a dynamic supportive structure for hematopoiesis. ECM proteins include fibronectin, collagen, vitronectin, and tenascin. Pertinent soluble growth factors include stem cell factor

(SCF), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). Present are also many adhesion molecules including members of the integrin superfamily.

MSCs constitute a small cell population residing within the stromal compartment of the bone marrow that can form other MSCs and generate mature cells of mesenchymal tissues. Current protocols of MSC isolation from bone marrow based on plastic adherence result in stromal cell cultures, not MSC cultures. In addition, many mesenchymal stem cell – like populations have been described in the literature, each having a unique phenotype. Some of these cell lines include bone marrow stromal stem cells (BMSSCs), stromal precursor cells (SPCs) and recycling stem cells (RS-1, RS-2). More recently, D'Ippolito *et. al.* [38] described human marrow-isolated adult multilineage inducible (MIAMI) cells which can proliferate extensively without signs of senescence or loss of differentiation potential. These cells may represent a more primitive subset of mesenchymal stem cell. Studies from Reyes *et. al.* [171] have also demonstrated multipotential adult progenitor cells (MAPC) with higher proliferative and differentiation potential. The varying phenotypes achieved from different research groups could be attributed to differences in isolation and culture protocols as well as types of media used for cultures. Whether different culture conditions favor growth of certain cell lines over others or cause a different phenotype for the same cell line is not known. These varying cell lines might also represent different points in the stem cell hierarchy [27]. Studies done by Lodie *et.*

al. [115] systematically compared different isolation and expansion protocols for adherent bone marrow cells and concluded that the resulting cells from all protocols where indistinguishable. In contrast, an unpublished study by Beyer Nardi and Da Silva reports a change in phenotype towards MAPCs when adherent bone marrow cells are cultured in MAPC culture conditions. Regardless, the varied results demonstrate that the mesenchymal stem cell compartment is heterogeneous and culture conditions can alter their basic properties.

Distribution Mesenchymal Stem Cells

The primary source of MSCs in adult individuals is the bone marrow stroma. Within the stroma, MSCs are present at a low frequency and recent studies suggest that in humans there is 1 MSC per 34,000 nucleated cells [216]. In mice, the frequency is an estimated 1 in 11,000-27,000 nucleated cells [132]. The heterogeneity of the bone marrow hampers adequate characterization and thus quantification of MSCs thus leaving many basic questions unanswered. MSCs have also been found in other tissues. In mice, MSCs have been isolated from brain, thymus, liver, spleen, kidney, muscle, and lungs. Other studies have also isolated MSCs from skin. Beyer Nardi and da Silva [10] attempt to explain this via three possible scenarios: (1) adult tissues contain independent reservoirs of stem cells, whose characteristic traits are determined by signals released by each niche; (2) MSCs exist as a reservoir in one specific location, from which they circulate through the organism to colonize different tissues; or (3) MSCs originate from blood vessel cell populations and are thus present throughout the whole organism.

This diversity in localization has prompted investigation into alternate tissue sources of MSCs.

Isolation and Culture of Mesenchymal Stem Cells

Few adult stem cells can be unequivocally identified and isolation requires in vitro or in vivo characterization via phenotype and functional traits (i.e. differentiation patterns). Unlike hematopoietic stem cells which have cell surface markers (CD34 in humans and Sca-1 in mice), MSC have no defined cell marker profile. Thus the isolation of MSCs has relied entirely on adherence to plastic surfaces [119,213].

To isolate MSCs, bone marrow is plated on plastic surfaces. Plastic adherent cells will adhere within 1 to 3 days, at which time, contaminant cells are removed via changing of the medium. The resulting culture is a heterogeneous stromal cell culture. Maintenance of this culture is through bi-weekly medium changes. The most widely used medium for these cultures is Dulbecco's modified Eagle's medium (DMEM), although others can be used. Cell cultures can be maintained for variable periods of time depending on species and organ of origin of the cells.

In an attempt to further purify this stem cell culture, immunodepletion has been attempted in order to eliminate hematopoietic cells displaying the markers CD45, CD34 and CD11b [103,153]. Other methods have involved size based filtering

of bone marrow cells through a 3-μm filter [93]. None of these methods have succeeded in yielding a pure MSC culture, thus the efficient and accurate isolation of MSCs remains an important goal for stem cell research.

Following the initial medium change, which removes all the non-adherent contaminants, cell culture lines are maintained with periodic passages until a relatively homogeneous population is achieved. As mentioned above, the most widely used medium is DMEM but others can be used and as a result can alter the phenotype of the cell culture. In addition to medium, supplementation with growth factors can alter the final phenotype of the culture line and are probably the main reason for variable mesenchymal stem cell phenotypes in the literature. For example, growth of murine MPACs depends on supplementation of leukocyte inhibitory factor (LIF) and the use of fibronectin-coated surfaces [97].

The ideal culture conditions would yield a stem cell culture maintaining MSCs with the phenotype and functional characteristics exhibited in their original niche, indefinite proliferation, and a capacity to differentiate into multiple cell lineages [10]. Since the first requirement is unknown, current culture strategies are focused on fulfilling the latter two requirements. The capacity of MSCs to proliferate without differentiation varies with species and culture mediums. Human MSCs have been shown to undergo 40 population doublings before a significant decrease in their growth rate is seen, where as murine MSCs show unlimited growth capacity without evidence of senescence [132]. The growth

capacity of human MSCs can be increased to 70 population doublings with the addition of supplemental growth factors, such as FGF-2 [11]. Expansion capacity of MSCs is also dependent upon seeding density. Human MSCs undergo more population doublings when plated at low density where as murine MSCs require a minimum of 2 x 10^6 cells/cm^2.

Homing and Engraftment of Transplanted Mesenchymal Stem Cells

The potential of MSCs to be used in gene and cell therapy relies intrinsically on the ability to target those cells to areas of degeneration or injury. Studies by Periera *et. al.* [159] demonstrated engraftment of murine MSCs in multiple organs following systemic injection. Successful engraftment of murine MSCs following intracranial injection was recently demonstrated by McBride et. al. [129]. This study also utilized an inter-species model whereby human MSCs were injected intraperitoneally into mouse embryos. Multi-organ engraftment resulted, as demonstrated with real-time PCR. The end organs analyzed within the study included femur, heart, brain, liver, kidney, spleen and lungs. Gojo *et. al.* [70] also demonstrated engraftment of murine MSCs to the heart, lung, spleen, stomach, small intestine, and skeletal muscle following intra-cardiac injection.

With multi-organ engraftment demonstrated, the question became would these cells preferentially engraft to damaged organs. Studies by Koplen *et. al.* [103] and Ortiz *et. al.* [153] demonstrated preferential MSC engraftment to damaged tissues following systemic injection. Both these studies demonstrated preferential

lung engraftment of MSCs in bleomycin induced lung injury mouse models. A study done by Gao *et. al.* [66] demonstrated preferential engraftment of MSCs to lung tissue in uninjured rats. This primary engraftment poses a potential problem as MSCs can become entrapped in lung capillaries resulting in fibrosis. This phenomenon was observed in the Gao study. The application of a vasodilator significantly decreased the amount of lung engraftment and improved engraftment to other organs including the liver and bone marrow stroma.

Although no engraftment studies have been performed in a human model, recent studies have demonstrated microchimerism present in women having gave birth to male fetuses [148]. This study tested the hypothesis that fetal MSCs traverse the placenta during pregnancy and engraft in maternal organs. The presence of XY containing MSCs were found in the bone marrow of these women. Engraftment of fetal MSCs to maternal thyroid, cervix, intestine, liver and lymph nodes has also been demonstrated [99]. The XY cells in these tissues were found to express liver, hematopoietic, or epithelial markers indicating tissue-specific incorporation.

Characterization of Mesenchymal Stem Cells

Extensive work has been done to date to characterize MSCs, in terms of morphology, cell surface and molecular markers. The markers that are sufficiently specific to select for these cells in culture have yet to be identified.

Thus, MSCs continue to be defined operationally, that is by their ability to self-renew, differentiate along various cell lines and adhere to plastic.

Numerous cell surface markers have been identified for human mesenchymal progenitor cells including CD44, CD29, and CD90. While the expression of CD34 is not well defined for murine MSCs, the absence of CD34 is an established marker for human and rat MSCs. Much of the work on cell surface markers has been done on human MSCs which are positive for Stro-1, SH2, SH3, SH4, MHC-1, and Sca-1 [8]. Majumdar *et. al.* [121] demonstrated a wide array of cell adhesion molecules expressed by hMSCs, which has provided insight into MSC engraftment and cellular interactions. Although many markers have been identified on MSCs, they lack sufficient specificity to function in purifying MSC cultures.

A review of MSC cell morphology revealed two main phenotypes: flat-type morphology and elongated, fibroblast like morphology. Nardi *et. al.* [10] summarizes various studies reporting the two morphologies. The flat-type morphology has been reported in rats, mice and humans while the elongated fibroblast-like morphology has been reported exclusively in humans.

Differentiation of Mesenchymal Stem Cells

In vitro differentiation of MSCs has been achieved using murine, rat and human MSCs. These cells can be differentiated along osteogenic, adipogenic,

chondrogenic or neurogenic cell lines (Fig. 1-6). Differentiation of these cells is confirmed with morphological changes and functional staining. For osteogenic differentiation, cells express elevated levels of alkaline phosphatase and produce a mineralized matrix which can be stained with osteocalcin. Chondrogenic differentiation results in the production of type II collagen, which can be stained. Neurogenic differentiation results in phenotypical changes resulting in a Schwann cell like morphology and expression of neuronal markers such as S-100 and GFAP[48] (Fig. 1-7).

In vitro, there is a great amount of heterogeneity in differentiation potential of MSCs. A hierarchy of pluripotency seems to exist whereby certain cells are less differentiated than others and thus are able to differentiate into more cell lines. Within a given cell culture, only a minority of MSC cells are truly pluripotent, with most of the cells being confined to bi- or uni- lineage differentiation capacity [49]. This limitation of differentiation might be a result of senescence, whereby cells loose differentiating capacity with each replicative cycle or passage. Another explanation is that the MSC cell cultures are not pure, with only a minority of cells present being true MSCs.

Mesenchymal Stem Cells in Peripheral Nerve Regeneration

With recent focus of peripheral nerve repair shifting from technical to biological advancements, the interest in growth factors, Schwann cells and now mesenchymal stem cell transplantation has been increasing. As discussed above,

A. **B.** **C.**

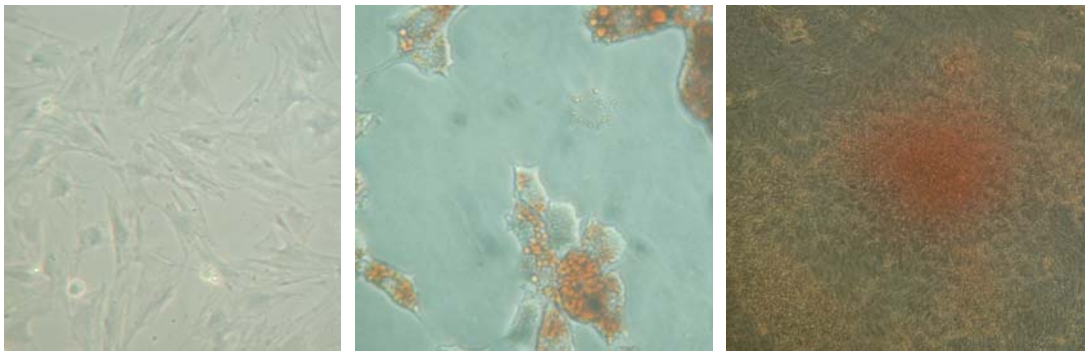

Figure 1-6: Differentiation of MSCs along mesodermal cell lineages. Undifferentiated MSCs demonstrate a large, flat, fibroblast morphology (A). Differentiation of MSCs towards an adipogenic lineage resulted in a change in morphology and development of lipid-laden vacuoles, which stained positive for Oil red O (B). Differentiation of MSCs along an osteogenic lineage resulted in the production of calcium matrix which stained positive for Alizarin red (C).

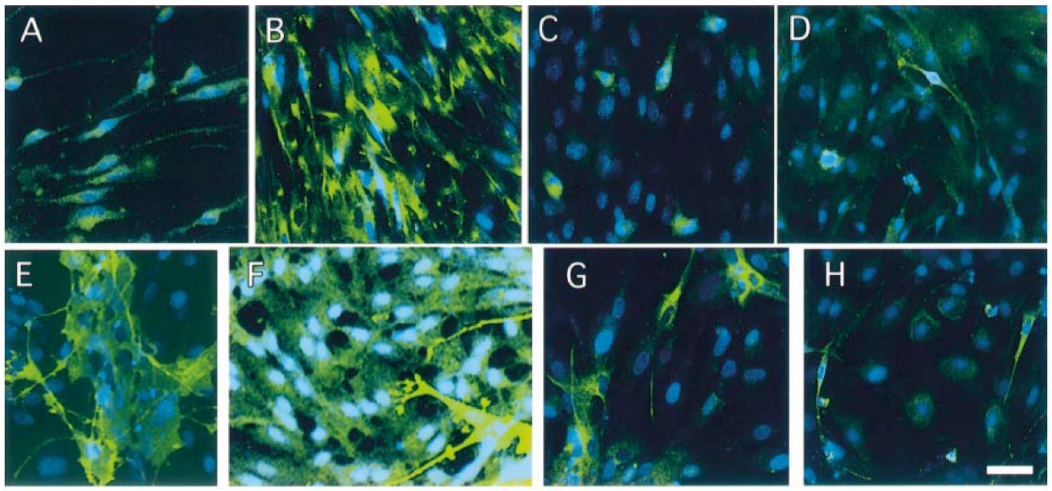

Figure 1-7: Immunocytochemical staining of Schwann cell markers p75 (A and E), S-100 (B and F), GFAP (C and G) and O4 (D and H) in differentiated MSCs (A±D) and Schwann cells (E±H). Scale bar, 50 mm.[48]

Schwann cell transplantation into peripheral nerve guides significantly improves quantitative, qualitative and functional outcomes in experimental models. With the recent recognition of the ability of MSCs to differentiate across embryological lineages, research has focused on the potential of MSCs to enhance peripheral nerve regeneration.

Transdifferentiation of Mesenchymal Stem Cells into Neuronal Phenotype

The differentiation of MSCs into a neuronal-like phenotype was first described by Woodbury *et. al.* [219], in both rat and human MSCs, where a simple differentiation protocol was used. A pre-induction medium containing beta-mercaptoethanol was followed by induction medium containing dimethylsulfoxide (DMSO) and butylated hydroxyanisole. This resulted in MSCs differentiating into a neuronal phenotype displaying a neuronal like morphology and expressing neuronal markers including neuron specific enolase [143], tau, and NeuN. When these differentiated MSCs where placed back into base media, they were observed to de-differentiate to the original MSC phenotype. Woodbury *et. al.* [218] and others have since modified this protocol to include bFGF which induces a more lasting differentiation effect. A study by Krampera *et. al.* [105] corroborated these findings by demonstrating neuronal differentiation of MSCs from human bone marrow, fat, spleen and thymus by using a modified differentiation protocol based on that originally described by Woodbury. The differentiation of MSCs into neurons holds great promise for the treatment of

neuro-degenerative disorders where neurons and synaptic connections are degenerating.

Transdifferentiation of Mesenchymal Stem Cells into Schwann Cell Phenotype

Peripheral nerve injuries present a different problem than central nervous system degenerative disorders. In these injuries, it is not the neuron that needs to be replaced, it is the axon that must regenerate and re-connect to target end organs. Thus, the replacement of neuron-like cells would be of marginal benefit. As described earlier, Schwann cells are vital for axonal regeneration and thus providing an enhanced source of Schwann cells to peripheral nerve injury sites would seem to be beneficial to axonal regeneration. Recent studies have demonstrated that MSCs can in fact differentiate into Schwann – like cells in vitro (Fig. 1-7). A differentiation protocol originally presented by Dezawa *et. al.* [48] involved pretreatment of the MSCs with beta-mercaptoethanol followed by treatment with bFGF, PDGF, Forskolin, and HRG. Further study by Dezawa resulted in the addition of Retinoic acid to the protocol. Beta-mercaptoethanol (BME) is a reducing agent which has been shown to induce morphological changes in MSCs[46,219]. Retinoic acid (RA) is a morphogen which has been reported to induce differentiation of embryonic stem cells and neural progenitor cells into nerve cells. BME and RA are thought to work as triggering factors, inducing changes in the morphological and transcriptional characteristics of MSCs [47]. FGF, PDGF, and Heregulin (HRG) have been shown to effect differentiation and proliferation in Schwann cells [95]. HRG, in particular, has been

shown to promote Schwann cell myelination [128]. Forskolin works by increasing the mitotic rate by increasing cAMP levels. It is theorized that all these factors work synergistically to induce differentiation of MSCs into a Schwann cell-like phenotype as omission of any one of these factors results in incomplete differentiation of MSCs as measured by morphological and immunoreactivity markers of Schwann cells. Markers for Schwann cells include S-100, GFAP, p75 and O4 [140].

Mesenchymal Stem Cells in Peripheral Nerve Regeneration

MSCs have been studied both *in vitro* and *in vivo.* *In vitro* differentiation of these cells has been achieved as described above but novel co-culturing techniques have allowed for the investigation of the effect of these stem cells on neuronal cells. A study by Caddick *et. al.* [25] examined the effect of undifferentiated stem cells, differentiated stem cells and Schwann cells on dorsal root ganglion cells *in vitro.* Co-cultures with Schwann cells and DRG cells demonstrated arborous type axonal growth and branching. Co-cultures of DRG cells with MSCs differentiated into Schwann-like cells demonstrated a more linear and longitudinal growth pattern. Co-cultures using undifferentiated stem cells resulted in minor axonal growth.

With *in vitro* evidence for the plasticity of MSCs towards the neuronal phenotype and *in vitro* evidence for the promotion of axonal outgrowth, recent investigations have focused on *in vivo* application to peripheral nerve injury

models. Dezawa *et. al.*[48] transplanted MSCs which had been differentiated in vitro into Schwann – like cells, to a nerve conduit to be used to bridge a sciatic nerve gap in a rat model. Green fluorescence labeling with confocal microscopy revealed MSCs surrounding newly formed axons and associated with newly formed myelin confirming in vivo Schwann cell like function of the MSCs. The study compared nerve regeneration between transplanted differentiated MSCs, non-differentiated MSCs and control acellular conduits. Results revealed a significant improvement in regenerating axon number and diameter in the differentiated MSC group as compared to the other two groups. The undifferentiated MSC group showed a modest improvement over the control. No functional analysis was performed.

Cuevas *et. al.* [35,36] demonstrated significant functional improvement, via walking track analysis and sciatic nerve index (SFI), when undifferentiated MSCs where injected directly into the primary repair site in a rat sciatic nerve injury model. Immunohistochemistry confirmed *in vivo* differentiation of the injected MSCs into Schwann cell-like phenotype. Building on initial histological studies by Dezawa, Mimura *et. al.* [135] demonstrated improved functional recovery with the incorporation of differentiated MSCs into an artificial nerve conduit in a rat sciatic nerve injury model. Similar results have been demonstrated in the mouse model [158].

Although emerging studies are indicative of a beneficial effect of MSCs in peripheral nerve regeneration, there exists a controversy to the benefit of differentiated MSCs as compared to undifferentiated MSCs. A recent study by Keilhoff *et. al.* [98] compared the efficacy of the incorporation of autologous Schwann cells, differentiated MSCs and undifferentiated MSCs into acellular muscle conduits to bridge a sciatic nerve defect. Contrary to Cuevas, Keilhoff *et. al.* demonstrated minimal functional benefit when using undifferentiated MSCs compared to differentiated MSCs and autologous Schwann cells. The minimal benefit of undifferentiated MSCs presented by Keilhoff could possibly be attributed to failure of *in vivo* differentiation. In a study by Tohill *et. al.* [200] undifferentiated MSCs incorporated into a nerve conduit and used to bridge a sciatic nerve defect were shown to express Schwann cell markers, indicating successful *in vivo* differentiation. The discrepancy might be explained by the actual percentage of cells differentiating and the sustainability of that differentiation.

The application of MSCs in peripheral nerve injuries has expanded beyond the use of bone marrow as a stem cell source. Pan *et. al.* [154] demonstrated improved functional outcomes with the incorporation of human amniotic-derived MSCs into a cellulose gauze to bridge a rat sciatic nerve defect. Most recently, skin-derived MSCs have been successfully incorporated into artificial nerve conduits with improved axonal regeneration and functional outcomes [123].

The effect of MSCs in peripheral nerve injuries is theorized to be supportive in nature and two fold. MSCs are believed to act like Schwann cells in that they function to prevent neuronal cell death and promote directional axonal growth. Studies described above demonstrate in vitro and in vivo differentiation of MSCs into a Schwann cell – like phenotype, but this differentiation has been shown to be reversible, where differentiated MSCs revert back to the MSC phenotype when returned to basal stem cell medium [48,98]. This phenomenon could, in part, explain the heterogeneity of results when analyzing the effect of MSCs on peripheral nerve regeneration. A fundamental obstacle faced by regenerating neurons is sustainability of a supportive environment, one that provides adequate neurotrophic and neurotropic factors. As this environment degrades, so might the ability of differentiated MSCs to maintain their differentiated state. A similar reversion to stem cell phenotype as seen *in vitro* could occur with the loss of growth supportive environmental cues. Thus, an additional obstacle to the use of MSCs is sustainability of a growth supportive phenotype. Other factors possibly attributing to varied results in the literature are differences in experimental techniques including nerve gap length, regenerative time, transplantation technique, and type of nerve guide used [98].

In Vivo Delivery of Mesenchymal Stem Cells

Should stem cells ultimately prove to be beneficial to functional recovery and indicated for clinical application, the question of how to best target these cells becomes an important one. To date, the most commonly sited method has been to

incorporate MSCs into artificial nerve conduits – both silicone and new biodegradable nerve guides [84]. These have proven to be effective in enhancing neuronal regeneration. Of note, the porosity of the nerve guide is vital to allow for intrinsic inflammatory cells to enter the site of repair. Another method of delivery utilized by Cuevas *et. al.* [35] directly injected the stem cells to the site of primary repair. This seems to be the simplest method of delivery but is limited to clinical situations where primary coaptation is possible. A study done by Pan *et. al.* [154] examined the incorporation of amniotic derived MSC into cellulose gauze and placing this gauze between the nerve ends with no formal coaptation. This study showed augmentation of nerve regeneration compared to using cellulose gauze alone. From these studies, it would seem that the MSC provide benefit through neurotrophic factor elaboration, but it remains unclear whether or not they have a neurotropic effect. The simplest way to test this would be to apply a matrix (capable of supporting MSC growth and migration) embedded with MSC stem cells directly into a nerve gap and monitoring regeneration. An additional benefit of this is minimization of iatrogenic trauma to the damaged nerve ends. No such study has been done to date.

Fibrin glue is a biologic adhesive originally marketed for hemostatic purposes. Its use has expanded to other applications including adhesive for nerve coaptations in place of sutures. It has received attention of late due to its ability to sustain MSC proliferation and migration [9,29,89]. Combined with its adhesive properties and solid composition, fibrin glue presents an effective method of MSC

delivery. *In vivo* study has successfully utilized fibrin glue as a delivery vehicle for MSCs to infracted myocardium [227]. Fibrin glue has also been used to target MSCs to cartilaginous and bony injury [29]. No literature was found using fibrin glue to target marrow derived MSCs to peripheral nerve injuries.

The concentrations of fibrinogen and thrombin have been shown to greatly influence the ability of MSCs to migrate within the fibrin matrix. In particular, the concentration of the fibrin component of the fibrin glue had the greatest influence on MSC migration. In vitro studies performed by Bensaid *et. al.* [9] determined the ideal concentrations of fibrinogen and thrombin to be 18mg/mL and 100IU/mL respectively.

Fibrin glue is an attractive vehicle for cell delivery in nerve injury for many reasons. Fibrin glue is simple to use and can be used as a coaptation device as well as a cell delivery device in peripheral nerve injuries. It also contains numerous growth factors which could intrinsically and independently exert an enhancing effect on nerve regeneration. Studies utilizing fibrin glue instead of suture coaptation have reported improved regeneration through coaptation sites with the glue [151,152].

Formulation of Thesis

A thorough review of the literature has revealed many advances in the understanding and treatment of peripheral nerve injuries. Both technical and

biologic advancements in nerve repair have capitalized on specific pathophysiological characteristics of nerve injury and regeneration. Despite numerous advancements, functional recovery following peripheral nerve injury remains poor. The reasons for this are multifactorial including mechanical impediment from fibrosis and scarring, inhibitory environmental elements including myelin debris and GAGs, inadequate trophic support, and inadequate tropic support. The purpose of this thesis will be to explore pharmacological, surgical and biological approaches to the treatment of peripheral nerve injury.

This thesis will be composed of the following papers:

I. Side-to-Side Nerve Grafts Sustain Chronically Denervated Peripheral Nerve Pathways During Axon Regeneration and Result in Improved Functional Reinnervation

Peripheral nerve injuries resulting in nerve transection or a nerve gap require surgical repair and are traditionally treated using end-to-end nerve coaptation with or without an intervening nerve graft. However, there are situations where such an approach is either unavailable or insufficient, such as in proximal nerve injuries. In these situations, alternative surgical methods can provide protection and support to chronically denervated nerve segments. The purpose of this paper was to present a novel technique in which lateral nerve connections in the form of side-to-side nerve bridges are used to support chronically denervated nerve pathways.

II. *In Vitro* Differentiation of Marrow Derived Mesenchymal Stem Cells Results in Expression of Schwann Cell Markers and Promotion of Axon Outgrowth

Nerve injuries resulting in a nerve gap require a graft to connect the severed proximal and distal nerve stumps. Currently, autologous nerve grafting is the treatment of choice, but it carries with it limitations including paucity of available nerve tissue and donor site morbidity with harvest of a nerve graft. Artificial nerve guides provide an appealing alternative to nerve grafting as donor site morbidity circumvented. Studies have shown that enhancing these conduits with Schwann cells greatly improves axonal regeneration through the conduit. However, like nerve grafting, harvesting Schwann cells requires the sacrifice of a nerve thus resulting in donor site morbidity. Recently, bone marrow derived MSCs have been shown to differentiate into a Schwann-like cell under specific culture conditions. To date, the differentiation process has not been adequately characterized and *in vivo* data on the effect on axonal outgrowth remains limited. The purpose of this study was to characterize the differentiation process quantitatively and temporally *in vitro*. Furthermore, this study aimed to investigate the histological and functional effects of these cells on axonal regeneration *in vivo*.

III. Rolipram-induced elevation of cAMP or chondroitinase ABC breakdown of inhibitory proteoglycans in the extracellular matrix promotes peripheral nerve regeneration

Pharmacological therapies to enhance axonal regeneration following peripheral nerve injury represent a simple treatment modality. As discussed, a major factor impeding axon regeneration in the PNS is the presence and delay in clearance of myelin debris and inhibitory GAGs. Rolipram and chondroitinase ABC have been investigated in the CNS, but a definitive role in peripheral nerve injury has yet to be established. In addition, the potential for combination therapy to theoretically achieve additive effects has yet to be investigated. The purpose of this study was to evaluate the effect of rolipram and chondroitinase ABC, both individually and in combination, on axonal regeneration following peripheral nerve injury.

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Chapter 2: Side-to-Side Nerve Grafts Sustain Chronically Denervated Peripheral Nerve Pathways During Axon Regeneration and Result in Improved Functional Reinnervation

Abstract

Alternative nerve repair techniques have focused on end-to-side neurorrhaphy as a sole means of repair of peripheral nerve injury or as a supportive adjunct in proximal nerve injuries. There is a narrow window of opportunity for regeneration following nerve injury such that in proximal nerve injuries, long-term neuronal axotomy and chronic denervation of distal nerve stumps will progressively reduce regenerative potential to almost zero. In this study we investigated the ability of side-to-side nerve bridges to induce and support axonal sprouting and regeneration distal to an injury site as a means to "protect" chronically denervated distal nerve pathways. Sprague Dawley rats (n=8) were divided into 2 groups, both of which underwent a unilateral transection of the common peroneal (CP) nerve. In group 1 (the unprotected group), the ends of the severed nerve were sutured back to muscle to prevent reinnervation. In group 2 (experimental group), in addition to suturing the ends of the CP nerve, three side-to-side nerve bridges (obtained from the contralateral CP nerve) were used to join the tibial (TIB) nerve to the distal stump of the CP nerve to promote axon regeneration into the chronically denervated CP nerve. Both groups were left to convalesce for 4 months and then the severed ends of the CP nerve in both groups were surgically repaired via primary suture coaptation. The rats were again allowed to convalesce for 5 months, at which time back-labeling was performed

using fluorescent retrograde dyes to quantify axonal regeneration. The mean number of motoneurons regenerating axons (±SE) regenerating through the CP nerve in the protected group (132.2±28) was significantly higher than in the unprotected group (76.7±33) ($p<0.05$), with a corresponding higher number of regenerated axon fiber count in the distal nerve stump. In addition to axonal counts, the mean tibialis anterior muscle weight (±SE) of the protected group (363.5±21 g) was significantly higher than in the unprotected group (219.3±29g) ($p<0.05$). Our data demonstrate the feasibility of using multiple side-to-side nerve bridges to maintain the integrity of a chronically denervated nerve and promote greater functional reinnervation following primary nerve coaptation of a chronically denervated nerve.

Introduction

Treatment of injuries to the nervous system presents a challenge due to the inherent complexity and topographic arrangement of nerve tissue. In addition, the limited regenerative capacity of the peripheral nervous system makes these injuries difficult to treat. The development of microsurgical techniques and a greater understanding of nerve physiology have resulted in improved outcomes, yet functional outcomes of nerve repair in the peripheral nervous system remain poor.

Traditionally, discontinuous nerve injuries have been treated with end-to-end nerve graft coaptation, which is currently the gold standard of nerve repair. When possible, this repair method has yielded satisfactory results. But, there are situations where such a repair option is unavailable or insufficient such as in proximal nerve injuries with long regenerative lengths, injuries where the proximal stump of the transected nerve is unavailable for coaptation, and injuries resulting in long nerve gaps. In proximal nerve injuries, for example, the current accepted method of treatment is cable grafting, but with such a proximal nerve injury, the time to regenerate to the end target muscle is often too long to achieve functional reinnervation[8]. This has been attributed to two main sequelae of nerve injury. First, the denervation of end-organ muscle results in disuse atrophy[9]. Second, the amount of time whereby the environment and resident Schwann cells remain conducive to regeneration is limited[28]. It has been shown in animal

models that axonal regeneration through nerve stumps that have been chronically denervated for 12 weeks approaches only 20% of normal[28] and thus alternative or additional supportive measures are required for proximal nerve injuries whereby the distance to the end muscle requires a prolonged time for regeneration to occur.

To overcome these problems, the concept of alternative surgical repair methods has emerged consisting of end-to-side and side-to-side nerve repairs. Viterbo *et. al.* revisited the end-to-side technique in 1992, which consists of taking the distal stump of a transected nerve and inserting it into a pre-formed epineural window of a donor nerve [33-35]. In their studies, they demonstrated both histologic and electrophysiologic reinnervation using end-to-side coaptation. A number of subsequent studies have been published in both experimental models and clinical cases studies, however the data for end-to-side nerve bridges to date show conflicting results [24,27]. Complicating the issue is the question as to whether true collateral sprouting occurs from nodes of Ranvier or whether donor axonal injury is required for axons to regenerate through the side-to-side bridge[1,13,23]. Regardless of the mechanism, axonal sprouting is essential for the success of this technique. From the literature, sprouting occurs more frequently in sensory axons as compared to motor axons[20,30].

In the human PNS, there exists supportive and redundant lateral connections between nerves, most notably in the upper limb between the ulnar and median nerves, namely Martin-Gruber and Riche-Cannieu anastomoses.

Based on these lateral topographic arrangements in the PNS, the side-to-side nerve repair technique was first described by Yuksel *et. al.* whereby epineural windows were created on the sides of a donor and recipient nerve and then coapted together in a lateral fashion. The study described a direct comparison between end-to-end, end-to-side and side-to-side nerve repairs[40]. Histological analysis and functional outcome measures indicated that the end-to-end repair resulted in the best regeneration followed by side-to-side nerve repair. End-to-side nerve repair yielded the least functional and histological regeneration. In 2004, a case report was presented whereby a high ulnar nerve laceration was repaired via end-to-end coaptation and supported distally by a side-to-side nerve bridge between the median and ulnar nerves[41]. Clinical follow-up revealed return of protective sensation as a result of the distal side-to-side nerve coaptation. Despite the promising results of these studies, there has been no further investigation in the current scientific literature evaluating the use of side-to-side nerve coaptation in injuries of the peripheral nervous system.

In clinical practice, situations present whereby traditional end-to-end nerve coaptation is insufficient to achieve an acceptable functional outcome and it is in these situations where alternative methods of nerve repair should be sought. With proximal nerve injuries, the ability to support primary end-to-end nerve grafting with a distal donation of axons is a concept that has been attempted with the use of end-to-side nerve bridges[5]. With traditional cable grafting, there often remains excess nerve that is discarded, which could be used as a side-to-side nerve graft,

bridging a donor nerve to a denervated nerve. Using the concept of side-to-side nerve coaptation, a side-to-side nerve bridge could be constructed between two nerves to function as a conduit for donor axonal growth. To date, such a technique has yet to be described. The purpose of this study is to evaluate the feasibility of using side-to-side nerve bridges as a means of supporting primary end-to-end coaptation in proximal nerve injuries. It is hypothesized that donor axons can propagate through side-to-side nerve conduits and as such can support functional regeneration by (i) supporting and maintaining the regenerative environment in the distal nerve stump and (ii) re-innervating end organs in a timely manner to provide protective sensation and circumvent disuse muscular atrophy.

Methods

All experiments were performed on adult female Sprague Dawley rats (250-275g) and approved by local authorities (Health Sciences Laboratory Animal Services, University of Alberta) according to the Canadian Council for Animal Care guidelines.

The efficacy of using fibrin glue for nerve coaptation

The side-to-side nerve bridges used in this study were coapted using Tisseel fibrin glue. To ensure that the use of fibrin glue was at least as effective as traditional suture coaptation, regeneration through end-to-end nerve coaptation was assessed with coaptation using either suture (9-0 nylon) or Tisseel fibrin glue (Baxter healthcare). Twelve Sprague-Dawley rats, with an average weight of

250g were used. Anesthesia was achieved using weight appropriate doses of Rompun and Ketamine (7mg/kg and 75mg/kg respectively). Under sterile micro-surgical technique, the right common peroneal (CP) nerve was exposed through a gluteal muscle splitting incision. The CP nerve was transected approximately 5mm distal to the sciatic bifurcation. At this point, the rats were divided into 2 groups (n=6, n=6). In group 1, the CP nerve was repaired via suture coaptation using 9-0 nylon suture. Silicone silastic guides (Helix Medical, Inc, Carpinteria, CA) of 0.76mm interior diameter and 3mm in length were implanted and the proximal and distal CP nerve stumps were approximated within the nerve guide using 9-0 Ethicon nylon suture (Ethicon, Inc Somerville, NJ, USA) [7]. In group 2, the CP nerve was repaired using Tisseel fibrin glue. The 2-component glue was applied and allowed to polymerize for 5 minutes, after which a 2-layer closure was performed. The rats were allowed to convalesce for 1 week, at which time, the nerves were back-labeled with retrograde fluorescent marker to quantify axonal regeneration across the suture sites.

Regeneration through side-to-side nerve bridges

The use of side-to-side nerve grafts to support axonal regeneration from a donor nerve to a recipient denervated nerve was investigated here. Twenty-two Sprague-Dawley rats, weighing between 250-275g, were divided into 2 groups (n=15, n=7). Anesthesia was achieved using weight appropriate doses of Rompun and Ketamine (7mg/kg and 75mg/kg respectively). Under sterile micro-surgical technique, the two major branches of the right sciatic nerve were exposed through

a gluteal muscle splitting incision. The common peroneal (CP) nerve was transected approximately 5mm distal to the point of bifurcation. The proximal and distal ends of the transected CP nerve were then sutured back to innervated muscle to prevent regeneration through the nerve ends[6]. In the first group of animals (n=15), a single epineural window was opened in the CP and tibial nerves. A 6mm CP nerve graft was harvested from the contralateral side and used to bridge the two epineural windows (Fig. 2-1A). In the second group of animals (n=7), three epineural windows were opened in the CP and tibial nerves. Three 6mm CP nerve grafts were harvested from the contralateral side and used to bridge the windows in the CP nerve to the donor tibial nerve (Fig. 2-1B).

Surgical protection of chronically denervated nerve by side-to-side nerve bridges

Eleven Sprague-Dawley rats, each weighing 250-275g were used in this part of the study. Anesthesia was achieved using weight appropriate doses of Rompun and Ketamine (7mg/kg and 75mg/kg respectively). The rats were split into 2 experimental groups (n=3 and n=8). Under sterile micro-surgical technique, the three major branches of the right sciatic nerve were exposed through a gluteal splitting incision. In the first group (n=3), the CP nerve was transected approximately 5mm distal to the point of bifurcation. The proximal and distal CP nerve stumps were sutured back to muscle to prevent spontaneous regeneration (Fig. 2-1C). A 2-layer closure was then performed and the animals were left to convalesce for 4 months. In the second group (n=8), an 18mm section of CP

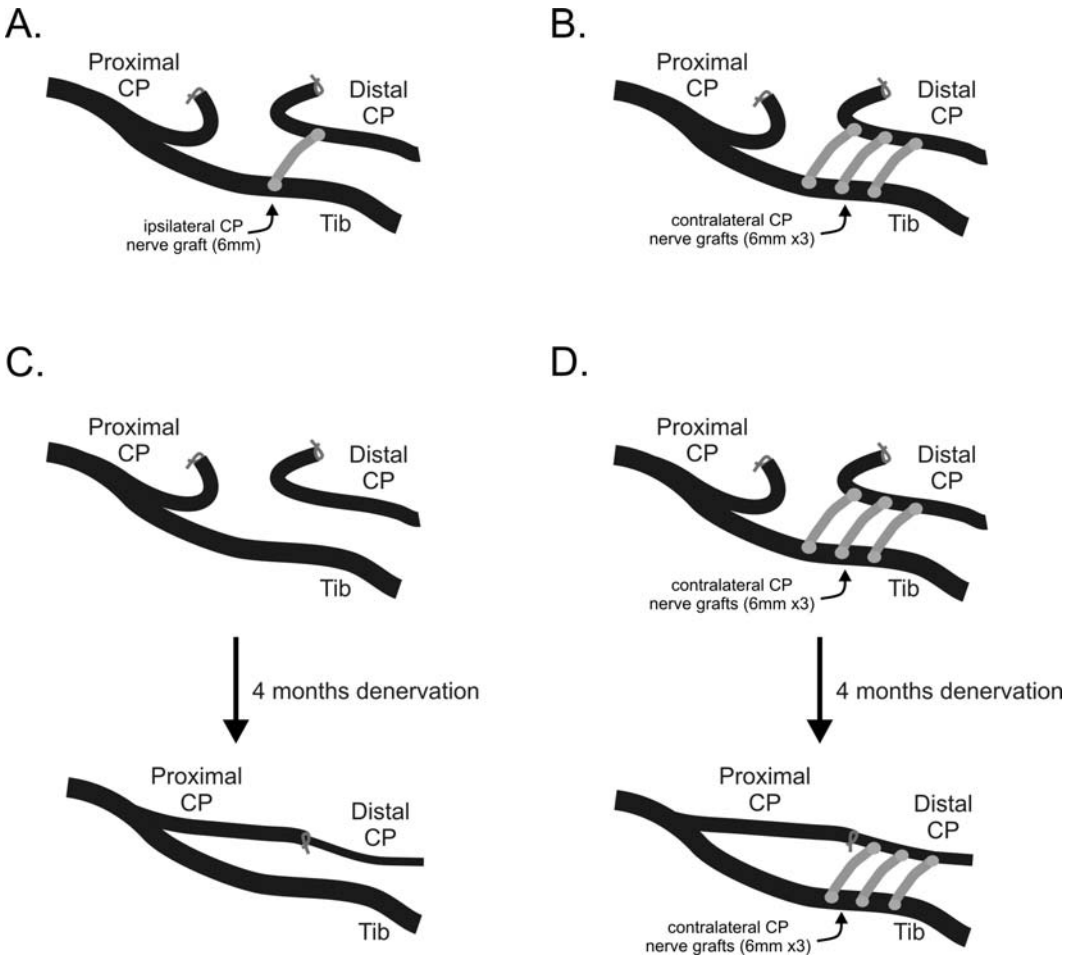


Figure 2-1: Single (A) and triple (B) side-to-side nerve grafts connecting the donor tibial nerve to the transected CP nerve. In the chronic denervation studies, the distal CP nerve stump was left denervated for 4 months without (C) and with (D) surgical protection in the form of 3 side-to-side nerve grafts joining the tibial nerve to the CP nerve.

nerve was excised from the left hind limb and cut into 6mm segments for use as side-to-side nerve grafts on the contra-lateral right hind limb. The left side was then closed via a 2-layer closure. The right sciatic bifurcation was subsequently exposed via gluteal muscle sparing incision. The CP nerve was identified and transected and the proximal and distal nerve stumps were sutured back to innervated biceps femoris muscle to prevent axon regeneration. Three epineural windows were then created on the distal CP nerve stump and on the tibial nerve. The three 6mm contralateral CP nerve grafts were then interposed between the epineural windows of the distal CP stump and the intact tibial nerve (Fig. 2-1D). Graft coaptation was achieved using Tisseel fibrin glue. A 2-layer closure was then performed and the animals were left to convalesce for 4 months. At 4 months, animals from both groups were anesthetized and the transected CP nerve was exposed. The proximal stump of the CP nerve was located and coapted to the distal CP nerve stump. The wounds were then closed and the animals were allowed to convalesce for 5 months, at which time, the animals were back-labeled and perfused as outlined below.

Back-labeling procedure using retrograde fluorescent dyes

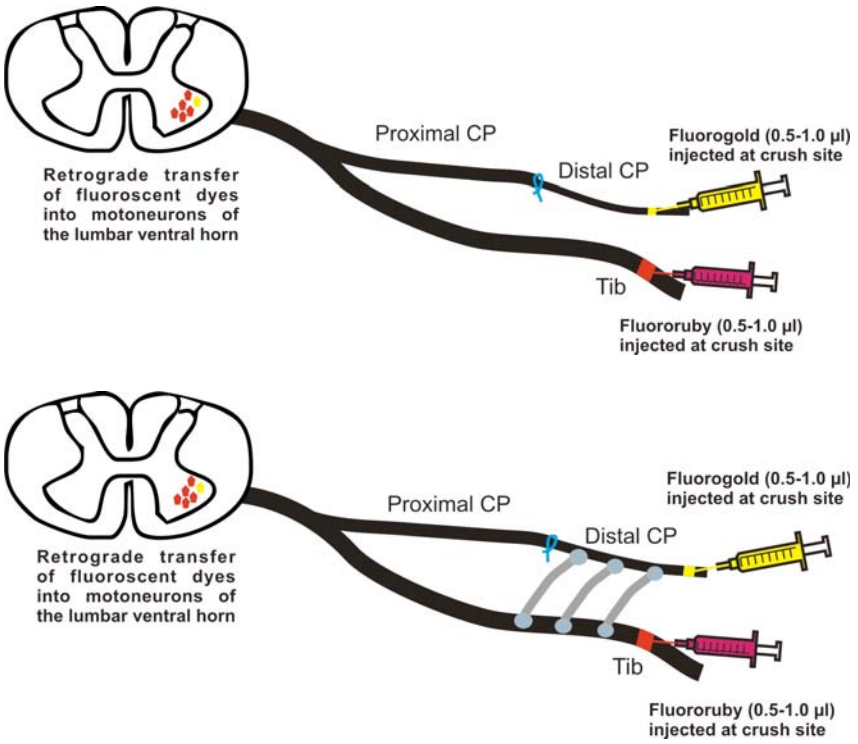
The animals were anesthetized and the right hind limb was opened through the previous incisions. The CP and tibial nerves were exposed and crushed 5mm distal to the most distal graft site and the crushed sites were filled with either fluororuby or fluorogold using a micropipette attached to a picospritzer (Intercel

Picospritzer III) (Fig. 2-2)[2]. The incisions were sutured and the animals were allowed to recover from anesthesia. One week following the back-labeling procedure, the animals underwent trans-cardiac perfusion. CP nerve segments were harvested for histology. The spinal cords were harvested for counting the motoneurons that successfully regenerated axons to the site of dye application (Fig. 2-2).

Morphological Analysis of Nerve Sections

At the time of neuron back-labeling, a 3mm segment of nerve was harvested just distal to the site of retrograde marker application. The nerve pieces were fixed in gluteraldehyde (3% in 0.1M cacodylate buffer) for 4 hours at 4˚C. The samples were post-fixed in OsO_4 (2%) for 2 hours, dehydrated through serial ethanol dehydration steps, and embedded in Epon resin. Transverse semi-thin sections (0.5 μm) of the entire nerve were stained with toluidine blue and examined under light microscopy. Images were obtained using an Olympus DP10 camera connected to a PowerMacintosh G3 computer and processed to a magnification of 200x for measurement of the cross-sectional area of the entire regenerated nerve, and to 2600x for myelinated axon counting. Randomly selected fields covering at least 25% of the cross-sectional area of the nerve were used to calculate the density of myelinated axons. The total number of axons was estimated from the density of myelinated fibers and the cross-sectional area of the regenerating nerve.

A.



B.

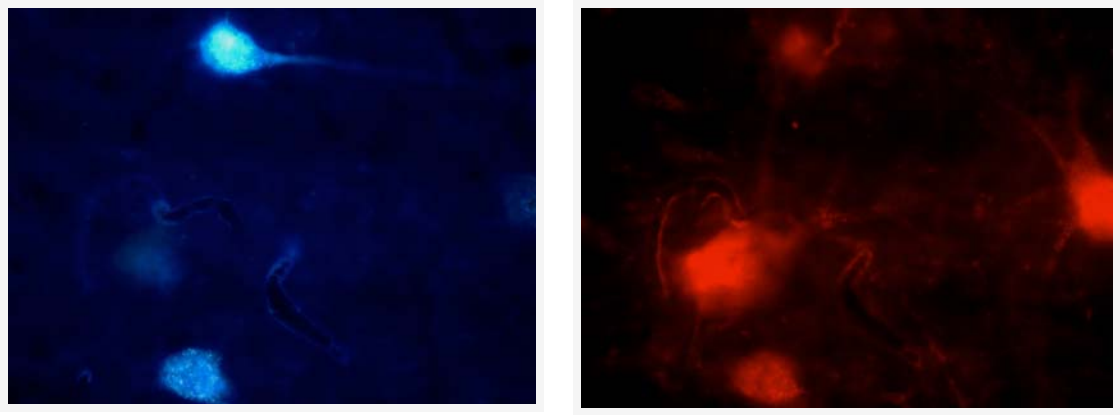


Figure 2-2: (A) Back-labeling procedure. Fluorogold and fluororuby injected into crush sites created 5mm distal to the side-to-side nerve grafts. Dyes were transported by axons that had regenerated to that point and their corresponding cell bodies were labeled. (B) Sample photographs of motoneuron cell bodies with in ventral horn of the

spinal cord stained with fluorogold (left) and fluororuby (right).

Statistics

Statistical comparisons were performed using SPSS 14.0 software. Values were expressed as a mean ± standard error (SE). The non-paired student t-test was used for comparisons of experimental and control groups. When more than 2 groups were compared, ANOVA was used to determine statistical significance. Significance was accepted at 5% ($p<0.05$).

Results

Nerve coaptation with fibrin glue equally as effective as suture coaptation

The use of fibrin glue in peripheral nerve surgery is relatively new with preliminary studies indicating improved regeneration using fibrin glue for nerve coaptation compared to traditional suture coaptation[21,22]. In our study, all side-to-side nerve grafting was achieved through fibrin glue coaptation to maximize the efficiency of the procedure. To ensure that the fibrin glue was equally as effective as traditional suture coaptation, the two methods were compared. In comparing the two methods, there were no incidences of dehiscence of the coaptation. In addition, there was no statistically significant difference in mean number of motoneurons regenerating axons across the coaptation site indicating that nerve coaptation using fibrin glue was equally as effective as suture coaptation (Fig. 2-3). In the suture group, the mean number of motor neurons regenerating axons across the coaptation (±SE) was 358±61 compared to 313±102 in the Tisseel fibrin glue group ($p>0.05$). In terms of ease and efficiency, application of the fibrin glue was technically more simple than suture coaptation. Due to the setting

time required for the 2-component glue, surgical times were similar between the two groups.

Significant axonal regeneration achieved through three side-to-side nerve bridges

The feasibility of using a nerve graft to bridge two nerves in a lateral fashion was investigated. Current literature on end-to-side nerve grafts suggests that disruption of the epineurium and donor axons is required to promote axonal sprouting into the graft[1,13], however numerous studies report collateral sprouting in the absence of donor axon injury[18,38]. Based on these observations for end-to-side coaptation, we hypothesized that increasing the area of epineural damage should increase the amount of axonal sprouting in the donor nerve. To evaluate this, regeneration through a single side-to-side nerve bridge and through three side-to-side coaptations were assessed using quantitative retrograde fluorescent marker labeling.

In performing lateral connections through side-to-side nerve bridges between the donor tibial and denervated CP nerve, increasing the amount of epineural damage and number of graft contact points significantly increased the amount of motor axon regeneration through the side-to-side nerve bridges (Fig. 2-4). When a single lateral nerve graft was used to join the donor tibial nerve to the denervated CP nerve, there was a minimal number of tibial motor neurons that regenerated their axons through the graft into the denervated CP nerve (5.7±2.1).

In contrast, when three side-to-side nerve bridges were used, a significant increase in number of tibial motoneurons regenerating their axons through the lateral nerve grafts was observed (48.4±18.9) ($p<0.05$). This value is quite small in comparison to the mean number of motoneurons regenerating axons through an end-to-end CP nerve graft of 6mm which was 290.5±9.6. A back-labeled intact CP nerve has 339±19 motor neurons. Thus, the use of three side-to-side nerve bridges resulted in 15% of normal motor neurons. Donor nerve morbidity is an important consideration when using alternative nerve repair techniques. Consistent with the controversy in the need for axonal damage to induce sprouting, is the occurrence of donor nerve morbidity with the creation of epineural windows. As described above, more extensive epineural damage resulted in improved regeneration through the side-to-side nerve bridges, but did this correspond to an increase in donor nerve morbidity? To address this, the donor tibial nerve was back-labeled with retrograde markers. Tibial motoneuron counts were not significantly altered with the creation of 0, 1 or 3 epineural windows with motoneuron counts of 583±13, 530±40.3, 594±46.9 respectively (Fig. 2-5). Thus, significant increases in axonal regeneration through the three side-to-side nerve grafts was achieved with no apparent decrease in tibial motoneuron count.

Side-to-side nerve grafts protect chronically denervated pathways.

Regeneration through side-to-side nerve grafts result in approximately 15% of regeneration seen using end-to-end nerve coaptation and thus at present

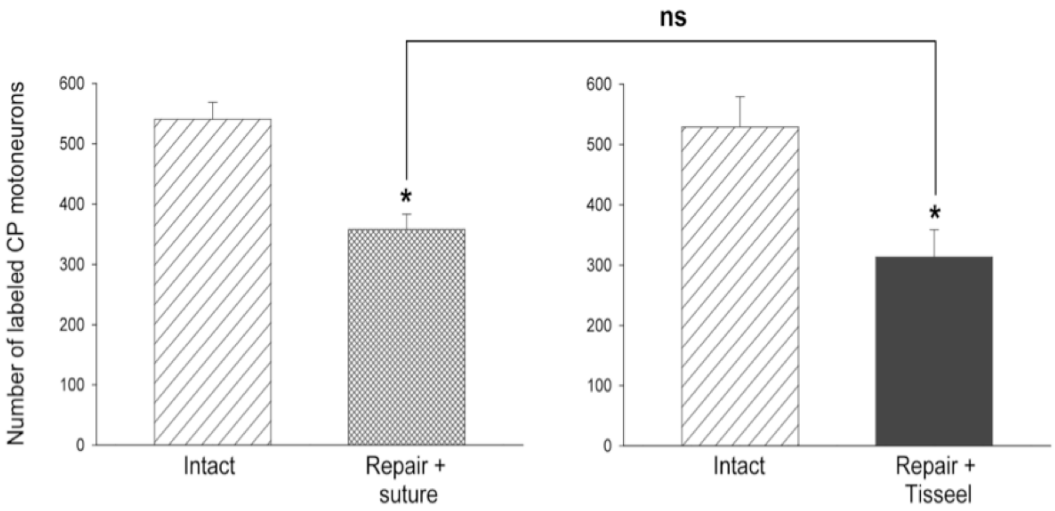


Figure 2-3: Surgical nerve repair was compared using Tisseel fibrin glue and suture coaptation of a transected CP nerve (n=6 suture; n=6 glue). The number of motoneurons regenerating axons across the coaptation site was equivalent regardless of whether suture or fibrin glue was used (*$p<0.05$).

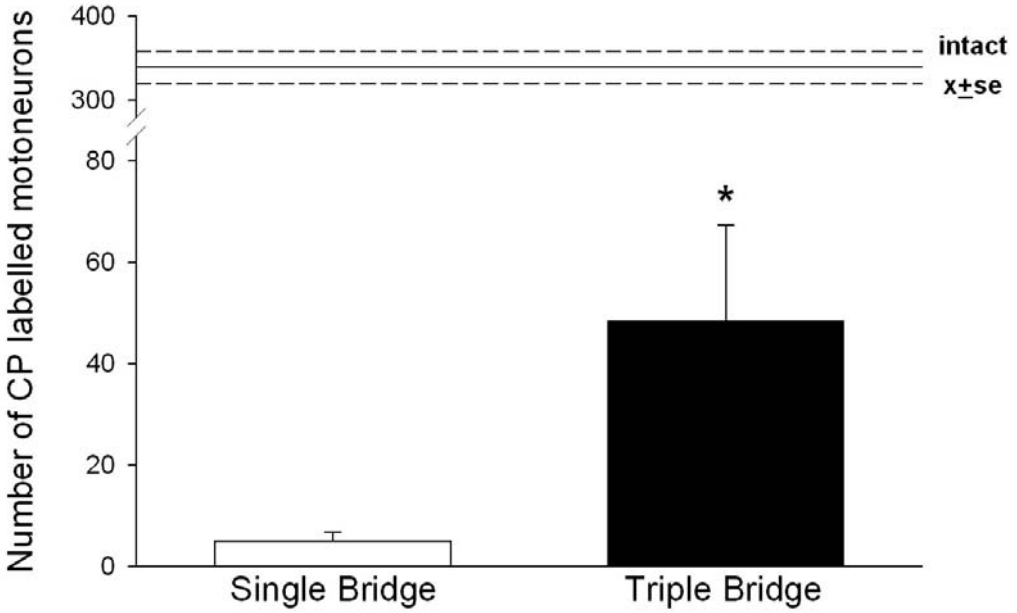


Figure 2-4: The number of tibial motoneurons regenerating axons through the side-to-side bridges into the denervated CP nerve stump significantly increased with the use of three bridges as opposed to a single nerve bridge ($p<0.05$) (n=15 single; n=7 triple). There was a 9.5 fold increase with the creation of 3 epineural windows with corresponding lateral connecting grafts compared to the creation of a single epineural window and graft.

cannot function as a sole repair method. However, the true role for these methods is as a supportive adjunct to primary nerve coaptation where the injury is proximal with a long regenerative distance. Use of side-to-side nerve grafts distally to provide regenerating axons to a chronically denervated nerve stump as seen in the distal limbs of proximal nerve injuries, can potentially function to sustain an environment supportive of axon regeneration within the denervated nerve conduit. Sulaiman *et. al.*[28] demonstrated that regenerative potential of a denervated nerve decreases with time. The purpose of this limb of the study was to evaluate the ability of side-to-side nerve bridges to provide axons to a denervated nerve from a donor nerve in order to sustain an environment supportive of regenerating axons proximally and to donate some axons for reinnervation of muscle to prevent disuse atrophy.

Protecting the a denervated distal CP nerve stump with three side-to-side nerve grafts results in increased motor axon regeneration through the CP nerve following delayed primary repair (Figure 2-6). With no side-to-side nerve graft, delayed primary end-to-end coaptation of the CP nerve resulted in a mean (±SE) number of 76.7±33 motoneurons, while protecting the distal CP nerve stump resulted in a significant increase in motoneuron regeneration (132.2±28) ($p<0.05$).

In addition to retrograde back-labeling methods, histological analysis of nerve segments at the site of back-labeling was used to assess the number of myelinated axons that had regenerated through the repaired denervated CP nerve.

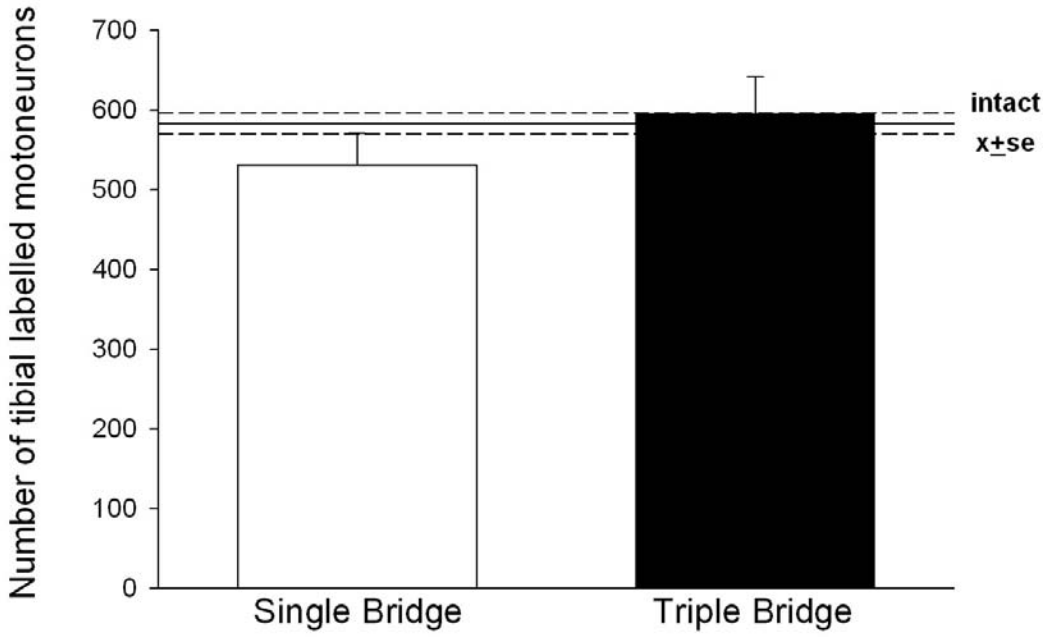


Figure 2-5: The number of tibial motoneurons labeled distal to the site of side-to-side coaptation in the tibial nerve were unchanged whether a single or three epineural windows were created ($p>0.05$) and both of which were not significantly different than the number of tibial motoneurons labeled in an intact tibial nerve (n=15 single; n=7 triple). Thus, minimal donor nerve damage was elicited with the creation of epineural windows for side-to-side coaptation.

Grossly, the protected CP nerve had larger and more densely packed myelinated fibers compared to the non-protected group (Fig. 2-7). In addition, the overall nerve diameter was larger in the protected group compared to the non-protected group, with well defined fascicular organization. Quantitatively, there was a statistically significant increase in number of myelinated nerve fibers in the group protected with side-to-side nerve bridges (2710±312) compared to the unprotected group. Similarly, when analyzing fiber density, there was a statistically significant increase in fiber density in the protected group (29.43±2.7 $*10^3$axons/mm^2) compared to the unprotected group (Fig. 2-8).

Tibialis anterior muscle weights were significantly increased when side-to-side nerve bridges were used to protect the chronically denervated CP nerve. Tibialis anterior muscle weights were used as an indirect measure of muscle reinnervation. With surgical protection, the mean tibialis muscle weight (±SE) was 363.5±21 grams compared to 219.3±29 grams in the unprotected group, yielding a 1.6 fold increase in weight ($p<0.05$) (Fig. 2-9).

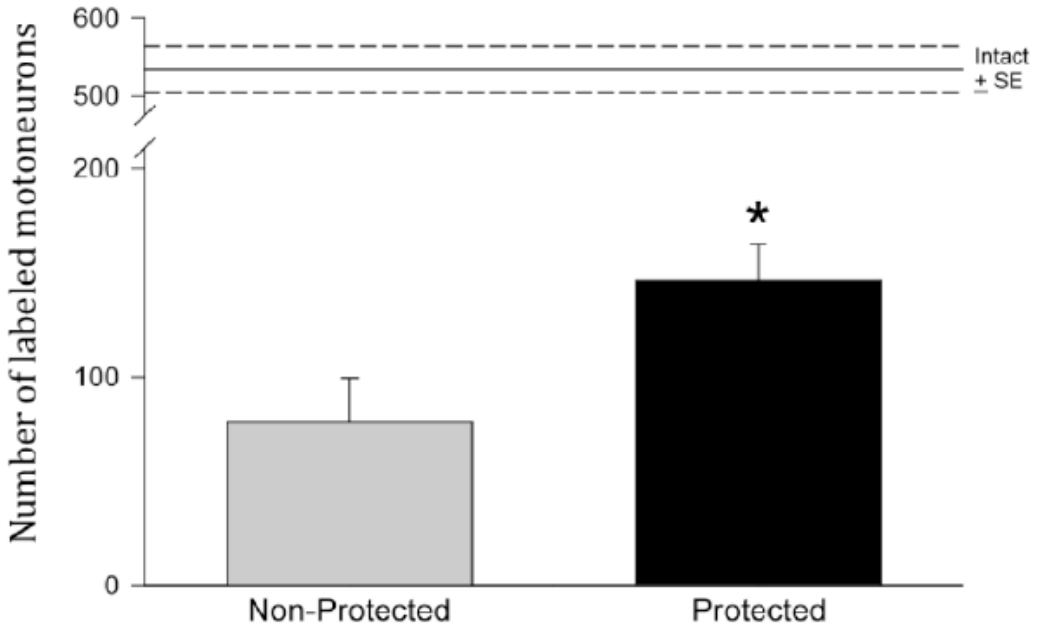
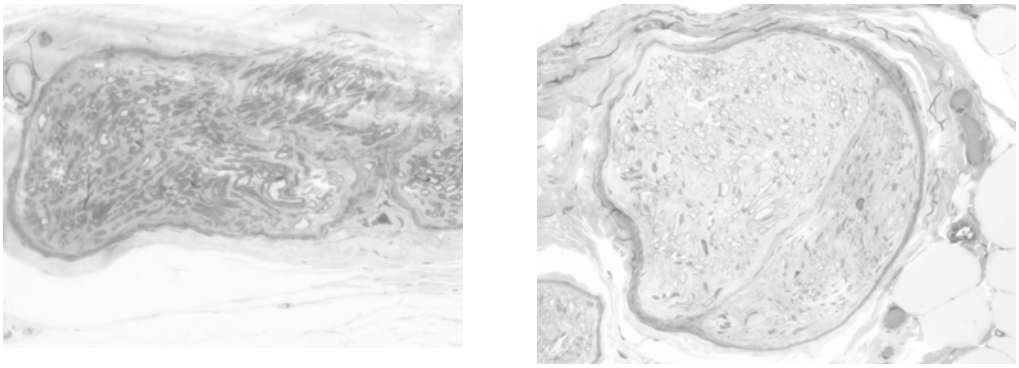


Figure 2-6: The number of motoneurons regenerating axons into the denervated distal CP nerve stump was significantly higher when the CP nerve was protected with three side-to-side nerve bridges, resulting in a 2-fold increase in number of motoneurons, equaling 40% of motoneurons labeled in an intact CP nerve (*$p<0.05$) (n=3 non-protected; n=8 protected). The number of labeled motoneurons in the non-protected denervated CP stump reached 20% of an intact CP nerve, consisted with previously published data by Sulaiman *et. al.*

A. **B.**



C. **D.**

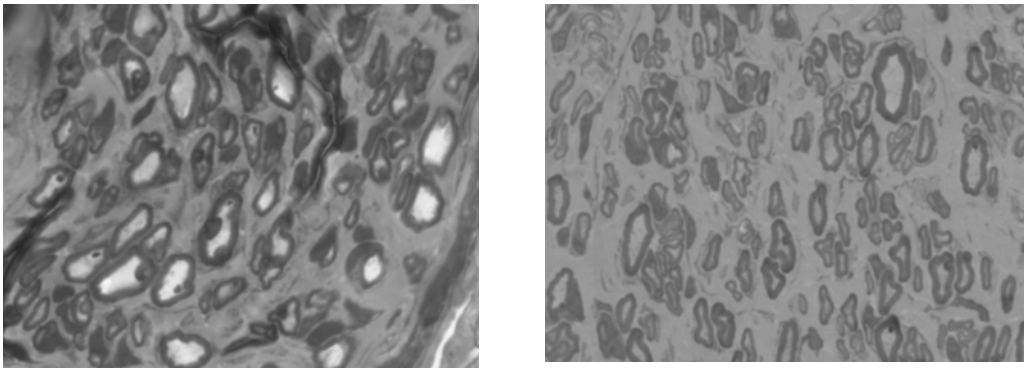


Figure 2-7: Histological cross-sections of nerve harvested 5mm distal to side-to-side nerve graft site. The non-protected distal CP nerve stumps demonstrated poor organization of fascicles and lower numbers of myelinated axon fibers (A, C) compared to the protected CP nerve stumps which had organized fascicles and higher numbers of myelinated axon fibers (B, D).

A. **B.**

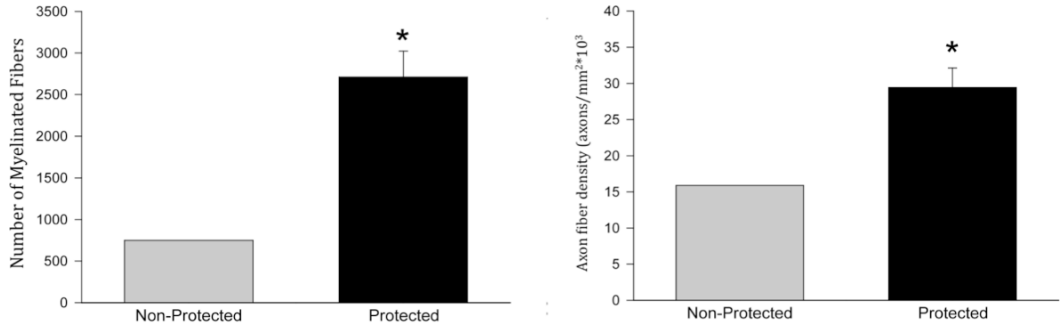


Figure 2-8: The number of myelinated nerve fibers present in the distal CP nerve stump when protected with three side-to-side nerve bridges was significantly greater than in the unprotected CP nerve (*$p<0.05$). Similarly, the axon fiber density in the CP nerve stump protected by lateral connections to the tibial nerve was significantly greater than in the unprotected CP nerve stump (*$p<0.05$).

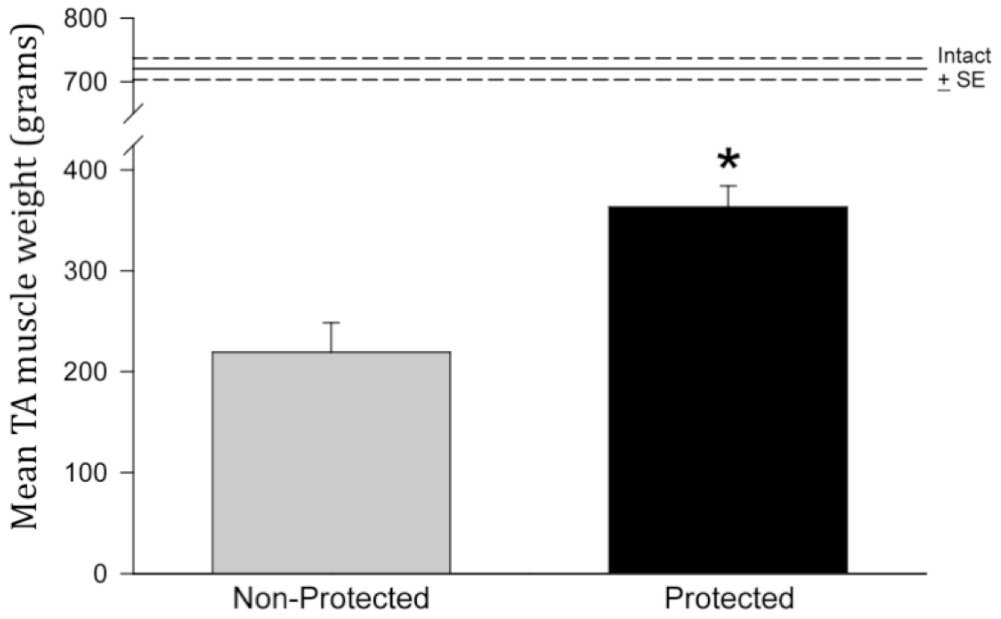


Figure 2-9: Tibialis anterior muscle weight was used as a measure of functional reinnervation. When comparing the tibialis muscle weights, surgical protection of the denervated CP nerve resulted in a significant increase in muscle weight and thus functional reinnervation compared to the non-protected group (*$p<0.05$).

Discussion

Functional recovery from peripheral nerve injuries remains a significant problem. While end-to-end nerve coaptation is currently the gold standard for nerve transection injuries, there are situations where such a repair option is unavailable or insufficient such as in proximal nerve injuries with long regenerative lengths, injuries where the proximal stump of the transected nerve is unavailable for coaptation, and injuries resulting in long nerve gaps. It is in these situations that alternative nerve repair methods should be sought out to increase chances of successful reinnervation and recovery. Improvements in regeneration can be achieved through minimizing Schwann cell denervation within a transected nerve segment and providing expedient reinnervation of muscle, thereby preventing disuse atrophy. In this study, we present a novel technique through which a nerve graft can be used to form lateral connections between an intact donor nerve and a denervated distal stump.

Axonal sprouting occurs in two forms: regenerative and collateral. In regenerative sprouting, axons extend branches in response to injury where as in true collateral sprouting, de novo branching of axons occurs at nodes of Ranvier [29]. In end-to-side neurorraphy, many studies have demonstrated that some degree of axonal injury is required to produce regeneration into the recipient nerve[1,4,13,17]. True collateral sprouting has been reported with some studies demonstrating axonal sprouting into end-to-side coaptations[18,38]. This de novo regeneration has been attributed to neurotrophic effects of Schwann cells and growth factors[3,32,38].

In our study, when we performed a single side-to-side nerve bridge between the donor tibial nerve and the denervated CP nerve, we found minimal regeneration through the graft. Technically, the epineural windows were small and were delicately performed to minimize donor axon injury. When we next created three side-to-side nerve bridges, there was a 9.5 fold increase in motoneurons regenerating axons through the lateral nerve grafts. Technically, the epineural windows were made larger for each window and there were now three areas of epineural damage compared to one. This resultant increase in regeneration through lateral coaptations indicates that larger epineural windows and increased contact point through which to regenerate promotes a greater degree of axonal sprouting. In a study by Yan *et al*, superior regeneration through an end-to-side coaptation was achieved by increasing the size of the epineural window and thus the surface area of contact between the graft and donor nerve[39]. This resulted in superior functional reinnervation as compared to a single, conservative epineural window. Similarly in our study, increasing the contact between the donor axons and the side-to-side grafts, greatly increased the regeneration through the lateral coaptations.

Donor site morbidity is an important consideration when manipulating a normal donor nerve. Paralleling the debate as to whether axon injury in the donor nerve is necessary for axonal sprouting is the debate as to whether lateral coaptation results in functional deficits in the donor nerve. Studies reporting true collateral sprouting inherently describe no donor site axotomy or functional

deficit[14,18]. In studies reporting the necessity for donor site axotomy to produce regenerative sprouting, donor axon morbidity has been documented although the functional implications of this are not completely understood[13]. With minimal donor nerve injury, functional deficits would not be expected due to the inherent plasticity of regenerating motor units where injured terminal motor branches can reinnervate 5-8 times their normal number of endplates thereby circumventing functional muscle impairment despite significant donor nerve injury[25,26]. In our study, there was no significant injury to the donor tibial nerve with the creation of one or three epineural windows. This can be attributed to meticulous technique in creating the windows and the use of a non-injuring method coapting the side-to-side nerve grafts using fibrin glue. The regeneration through the lateral nerve connections was modest and according to the literature might be improved with more aggressive donor nerve injury, but the extent to which the donor nerve can be injured without functional impairment has yet to be elucidated. Unlike other studies using suture coaptation in combination with epineural window creation, the use of non-injuring techniques such as the use of fibrin glue for coaptation might create the optimum balance between injury and sprouting. From our preliminary study, regeneration through fibrin glue coaptation was equivalent to suture coaptation with no incidence of nerve dehiscence, which is consistent with the literature[19,21,22]. In addition, it is documented that collateral sprouting is induced by Schwann cells and growth factors including Insulin-like growth factor[3,18,32]. A potential added benefit of using fibrin glue for coaptation, which

has not been investigated to date, is the ability to enrich the compound with desired growth factors to further enhance axonal sprouting.

In proximal nerve injury, such as brachial plexus and high ulnar nerve injury, there is a long regenerative distance to denervated target. Functional recovery following these injuries is poor which has traditionally has been attributed to denervation atrophy proceeding to complete loss of muscle[8,9,16]. More recently, Fu and Gordon demonstrated that the principle mechanism for poor reinnervation following prolonged denervation is deterioration of the distal nerve rather than the inability of muscle to accept regenerating axons[6]. In their study, a freshly axotomized tibial nerve was cross-sutured into a denervated CP nerve stump. With increasing periods of denervation of the distal nerve stump, there was a dramatic decline in the number of motoneurons regenerating axons into the distal stump. This decline in reinnervation was temporally quantified in a subsequent study. Impairment in reinnervation was first observed at 8 weeks of denervation and progressively increased whereby at 3 months, regeneration reached 22% of normal and by 6 months post-injury, there was little to no regeneration into the distal denervated CP nerve stump[28]. It has been demonstrated that Schwann cells are required for efficient axonal regeneration to occur[10-12] and that Schwann cell numbers progressively decline with prolonged denervation[37]. The decline in Schwann cell number has been attributed to lack of axonal contact whereby chronic denervation leads to degradation of Schwann cell bands of Büngner leading to a decline in the ability of axons to regenerate into the

denervated nerve stump[36,37]. This pattern of Schwann cell and basal lamina degradation with chronic denervation observed in experimental models has been confirmed in human surgical specimens[31].

In this study, we attempted to protect chronically denervated distal nerve stumps by providing axonal contributions from a donor nerve in order to provide axonal contact to Schwann cells that would otherwise not survive. In creating side-to-side nerve bridges, donor axons from the tibial nerve regenerated into the denervated CP nerve serving to preserve axonal contact and thus the integrity of Schwann cell tubes within the distal CP nerve stump[15]. As anticipated, protecting the distal denervated CP nerve stump resulted in significant improvement in axonal regeneration into the chronically denervated CP nerve stump. From studies by Sulaiman *et. al.*, we expected the number of motoneurons regenerating axons into a distal nerve stump that has been denervated for 3 months to reach approximately 22% of normal[28]. This coincides with our control group, which yielded regeneration nearing 20% of normal. By protecting the denervated CP nerve stump, regeneration reached 40% of normal, a 2-fold increase. Similarly when examining the total number of myelinated nerve fibers, both motor and sensory, that regenerated into the chronically denervated CP nerve stump, there was a 3.3 fold increase with surgical protection of the denervated nerve. Coinciding with the increase in axon number, there was a 1.75 fold increase in axon fiber density within the chronically denervated CP nerve stump with the use of side-to-side nerve grafts distal to the site of injury. Histologically, fascicle

structure appears more organized and myelinated fibers appear larger with surgical protection. These results are consistent with the view that axonal contact is required to maintain Schwann cell and basal lamina integrity which are, in turn, needed for successful regeneration. Paralleling improved axonal regeneration was a significant increase in tibialis anterior muscle weight indicating improved functional muscle reinnervation with surgical protection of the denervated CP nerve.

The results of this study have significant translational implications. Clinically, in high ulnar nerve injury resulting in a nerve gap for example, a cable nerve graft is used to bridge the defect in an end-to-end fashion. Even with timely repair, the regenerative distance is sufficiently long that functional reinnervation is poor. From experimental data, it is likely that the Schwann cells and basal lamina in the distal stump of the ulnar nerve degrade thereby impeding axon regeneration. In such a situation, if the Schwann cells could be preserved, reinnervation would be enhanced and likely result in better functional outcomes. It is in this situation that the use of alternative nerve repair techniques such as side-to-side nerve bridges has clinical application. Often when creating a cable graft, there is nerve left over which could be used to form lateral connections between the median nerve and the denervated ulnar nerve distally in the forearm. This would serve to provide axonal contact and innervation to Schwann cells that would otherwise be denervated for a prolonged period of time. As seen in our animal model, surgical protection of these distal denervated nerve pathways can

lead to improved regeneration into the distal ulnar nerve and better functional reinnervation of muscle.

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Chapter 3: *In Vitro* Differentiation of Marrow Derived Mesenchymal Stem Cells Results in Expression of Schwann Cell Markers and Promotion of Axon Outgrowth

Abstract

Purpose: Treatment of injuries to the nervous system is a challenge due to the inherent complexity and topographic arrangement of nerve tissue. In addition, the limited regenerative capacity of the peripheral nervous system makes these injuries difficult to treat. Among the most exciting areas of research is the discovery of mesenchymal stem cells and their inherent plasticity allowing for differentiation into multiple cell lineages including chondrocytes, osteoblasts, and adipocytes. More recently, preliminary reports have described the differentiation of mesenchymal stem cells (MSC) into a Schwann cell phenotype. The purpose of the study was to quantitatively and temporally characterize MSC differentiation into a Schwann cell-like phenotype and to systematically evaluate the functional capacity of these cells to promote axon outgrowth *in vivo*.

Methods: MSCs were isolated from the bone marrow of Sprague-Dawley rats and were characterized based on plastic adherence and pluripotency towards mesodermal lineages. The isolated stem cells were then stimulated towards a Schwann cell phenotype using specific growth factors. Differentiation was characterized using immunocytochemical staining and flow cytometric analysis for Schwann cell markers at different time points. Additionally, the neurotrophic effect of these differentiated stem cells was assessed both *in vitro*, using an

indirect co-culture system with rat dorsal root ganglion (DRG) cells, and *in vivo* in a rat sciatic nerve gap model.

Results: The isolation of MSCs resulted in a mixed marrow population including MSCs and a mixed population of accessory cells. The purity of MSC culture increased with each subsequent passage. The differentiation procedure used to achieve the Schwann cell phenotype resulted in positive immunocytochemical staining for the Schwann cell markers glial fibrillary acidic protein (GFAP), S100, and nerve growth factor receptor (NGFR). Following 6 days of differentiation, FACS analysis of the differentiated stem cell population revealed 51% of cells expressed GFAP, 36% of cells expressed S100 and 45% of cells expressed NGFR. Interestingly, longer periods of *in vitro* differentiation (10 and 14 days) resulted in a progressively decreased proportion of cells expressing Schwann cell markers. Analysis of the co-cultures revealed neurite extension was greatest in the Schwann cell co-culture (0.32±0.019 mm) followed closely by the differentiated MSC (dMSC) co-culture (0.30±0.018 mm), which were both significantly greater than the undifferentiated MSC (uMSC) (0.22±0.015 mm) and DRG alone (0.13±0.02 mm) culture systems ($p<0.05$). *In vivo*, transplantation of dMSCs resulted in a greater number of motoneurons (627.75±86.02) extending axons across a nerve gap than an empty conduit (284.83±48.11); however, we did not observe any improvement in functional outcome measures including tibialis anterior muscle weight and EMG.

Conclusion: Bone marrow derived MSCs were successfully differentiated into a Schwann cell phenotype expressing Schwann cell markers however, *in vitro*, this differentiation appears to be transient, decreasing with time. Additionally, dMSCs provided support for axonal regeneration equivalent to that of Schwann cells both *in vitro* and *in vivo*.

Introduction

Peripheral nerve injuries result in significant impairment of sensory and motor function distal to the site of injury. These injuries, even if surgically repaired, often result in poor functional outcomes. This is attributed to a number of obstacles to regeneration including nerve gaps and limited time period in which Schwann cells remain supportive to axonal regeneration[20]. With advances in microsurgical technique, there has been much improvement in the treatment of peripheral nerve injuries; however, there are situations where simple primary coaptation of a transected nerve is not possible or insufficient for functional recovery. Clinically, these scenarios include proximal nerve injuries with long regenerative distances and injuries resulting in a nerve gap.

Currently, autologous nerve grafting is used to repair nerve transection injuries that result in a nerve gap. Autologous nerve provides an ideal basal lamina and a source of Schwann cells[35], which are both required to effectively bridge large nerve gaps[22,50]. Studies have shown than conduits containing Schwann cells support axonal regeneration more efficiently and over longer distances than grafts composed of basal lamina alone [22-25]. This has been attributed to the ability of Schwann cells to synthesize neurotrophic factors and basal lamina[21,35]. The presence of laminin and fibronectin in the basal lamina of the graft is beneficial to axon regeneration, antibodies to fibronectin and laminin having been shown to inhibit axonal regeneration through sciatic nerve grafts [50].

Autografts have long been used to bridge gaps in nerve continuity but are in limited supply and require sacrifice of an intact nerve, resulting in donor site morbidity. To circumvent this, focus has turned to using alternative nerve conduits to bridge nerve gaps, which have resulted in modest axonal regeneration, subsequently leading to interest in biological augmentation of alternative nerve conduits with growth factors and/or Schwann cells.

Schwann cells are the major supporting cell type in the peripheral nerve and play an integral role in peripheral nerve regeneration[3,30]. Following nerve injury, Wallerian degeneration proceeds at the distal nerve stump where Schwann cells function to to clear myelin debris and secrete growth factors with neurotrophic and neurotropic properties including nerve growth factor (NGF), neurotrophin 4/5, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) 1 and 2 and glial cell line derived neurotrophic factor (GDNF) [9,17,33]. In the absence of axonal contact, Schwann cells proliferate to fill the endoneurial shealth, forming longitundinal columns known as bands of Büngner, which function to accept regenerating axons from the proximal nerve stump[16,48]. Experimentally, transplantation of Schwann cells has been shown to enhance axon outgrowth both *in vitro*[44] and *in vivo*[2,31]. However, harvesting autologous Schwann cells requires the sacrifice of a donor nerve, leading to donor site morbidity and presenting a similar problem to autologous nerve grafting. Additionally, Schwann cells have limited growth

potential *in vitro*. These limitations have led to investigation of other cell types that may provide similar trophic support to regenerating axons.

Mesenchymal stem cells (MSCs) are a population of non-hematopoeitic multipotent somatic stem cells harbored in the bone marrow. They are defined by their ability to adhere to plastic surfaces and by a panel of positive and negative cell surface markers[41]. Under specific conditions, these cells can differentiate into multiple mesodermal cell lineages including chondrocytes, osteocytes and adipocytes[40,41]. More recently, MSCs have been shown to be able to differentiate into neuronal phenotypes including astrocytes, oligodendrocytes, microglia and neurons[1,10,43,51]. MSCs were first shown to be capable of differentiating into cells with Schwann cell properties by Dezawa *et. al.* [12] in 2001. Subsequent studies have demonstrated the expression of Schwann cell markers qualitatively through immunocytochemistry[12,31,49] with only one recent study attempting to quantify Schwann cell marker expression in differentiated MSCs[34]. Functional properties of these cells have been demonstrated *in vitro* where co-cultures of dorsal root ganglion cells with MSCs differentiated towards a Schwann cell phenotype result in increased neurite outgrowth[4]. Several studies have examined *in vivo* application of MSCs in an animal nerve injury model with preliminary results indicating improved axonal outgrowth histologically, although studies examining functional outcomes remain limited and inconsistent[12,31,37,39].

Studies to date have provided promising preliminary evidence for the transplantation of MSCs to promote axonal regeneration and functional reinnervation followng nerve injury. The actual efficiency of MSC differentiation and permanance of the differentiated phenotype require further investigation. In addition, functional benefit in *in vivo* models remains preliminary. The purpose of this study was to further characterize bone marrow derived MSCs, quantify terminal differentiation into a Schwann cell like phenotype and evaluate temporal phenotype permanence. It is hypothesized that by optimizing the differentiation process thereby increasing the yield of MSCs successfully differentiating into a Schwann cell phenotype, transplantation of these cells into a nerve gap will enhance axonal regeneration and functional reinnervation.

Methods

All experiments performed on animals were approved by local authorities (Health Sciences Laboratory Animal Services, University of Alberta) according to the Canadian Council for Animal Care guidelines.

Mesenchymal stem cell harvest and culture

Seven week old Sprague-Dawley rats (180-220g) were euthanized by an overdose of rompun and ketamine (7mg/kg and 75mg/kg respectively). The femur and tibial bones were isolated from the hind limbs, the ends cut, and the marrow flushed with 5mL of α-MEM supplemented with 10% fetal bovine serum

(FBS). The aspirate was filtered through a 70μm filter and centrifuged at 1,000 rpm. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 mcg/mL streptomycin. The cell suspension was then plated on 75cm^2 plastic flasks at a seeding density of 500 cells/cm^2. The cells were incubated at 37°C, 95% humidity and 5% CO_2 for 24 hours, at which time non-adherent cells were removed and the media was replaced. The adherent cells were labeled passage 0 (P0) and grown to 80% confluence.

When the cells reached 80% confluence, they were detached through incubation with 0.25% trypsin and 1mM EDTA for 5 minutes. The cells were harvested and sub-cultured such that one flask of cells was divided into two flasks. The media was changed every 3 days. P2 cells were used for all experiments.

Characterization of cultured MSCs

MSCs at P2 were characterized by evaluation of cell marker profile and through a mesodermal cell line differentiation assay. Cells in 75cm^2 plastic flasks were harvested using 0.25% trypsin and 1mM EDTA and then fixed in 4% paraformaldehyde (PFA) for 10 minutes. The cell suspension was washed with 1X phosphate buffered solution (PBS) three times and 1x10^5 cells were aliquoted into each of 6 flow cytometry tubes. The cells were incubated with antibodies to CD14, CD45, CD54, integrin-β1, and appropriate mouse and rabbit IgG controls

for 1 hour at room temperature. Following 3 washes with 1X PBS, the cells were incubated with FITC and TRITC secondary antibodies for 45 minutes at room temperature. The cells were washed 3 times in 1X PBS, post-fixed with 1% PFA and examined using FACSCalibur (BD Biosciences).

For the differentiation assay, P2 cells seeded in 6 well plates were induced to differentiate into osteogenic and adipogenic phenotypes using specific culture conditions[40,41]. For osteogenic differentiation, cells were incubated with α-MEM supplemented with 10% FBS, 0.1μM dexamethasone, 50μM ascorbate–2–phosphate, and 10mM β-glycerophosphate. For adipogenic differentiation, cells were incubated with α-MEM supplemented with 10% FBS, 1μM dexamethasone, 10μM insulin, 200μM indomethacin, and 0.5mM isobutyl-methylxanthine. Full media changes were performed every 3 days for 2 weeks, at which time staining of the cells was done with Alizarin red and Oil red O to confirm osteogenic and adipogenic differentiation respectively.

Transdifferentiation of MSCs into Schwann-like cells

P2 MSCs were induced to differentiate into Schwann-like cells according to a modified protocol from Dezawa *et. al.* [12]. Cells plated, at a seeding density of 500 cells/cm^2, on 75cm^2 plastic flasks and culture chamber slides were used for flow cytometry and immunocytochemical analysis respectively. After 3 days of incubation with α-MEM supplemented with 10% FBS, the media was changed to serum free α-MEM supplemented with 1mM β-mercaptoethanol. After a 24 hour

incubation, the media was changed to α-MEM supplemented with 10% FBS and 35 ng/mL of all-*trans*-retinoic acid. Following a 72 hour incubation, the cells were incubated with transdifferentiation media consisting of α-MEM supplemented with 10% FBS, 10ng/mL basic fibroblast growth factor (Chemicon, Billerica MA), 5ng/mL human recombinant platelet derived growth factor (Chemicon, Billerica, MA), 5μM forskolin (Sigma, St. Louis, MO), and 126 ng/mL glial growth factor-2 (Accorda Therapeutics, Hawthorne, NY). The cells were incubated in this media for 2, 6, 10 and 14 days with media changes every 2-3 days.

Characterization of differentiated mesenchymal stem cells

Schwann cell marker analysis was performed after incubation of MSCs in transdifferentiation media for 2, 6, 10 and 14 days using fluorescence-activated cell sorting (FACS) and immunocytochemistry. The Schwann cell markers used for this analysis were S100, glial fibrillary acidic protein (GFAP) and nerve growth factor receptor (NGFR). For FACS, cells in 75cm^2 plastic flasks were harvested using 0.25% trypsin and 1mM EDTA and then fixed in 4% paraformaldehyde (PFA) for 10 minutes. The cell suspension was washed with 1X phosphate buffered solution (PBS) three times and 1x10^5 cells were aliquoted into each flow cytometry tube. The cells were incubated with antibodies to S100 (Sigma, St. Louis, MO), GFAP (Chemicon, Billerica, MA), NGFR (Chemicon, Billerica, MA), and appropriate mouse and rabbit IgG controls for 1.5 hours at room temperature. Following three washes with 1X PBS, the cells were

incubated with FITC and TRITC secondary antibodies for 1 hour at room temperature. The cells were washed 3 times in 1X PBS, post-fixed with 1% PFA and examined using FACSCalibur.

For immunocytochemistry, differentiated cells cultured in 4-well chamber slides were fixed with 4% PFA for 10 minutes. The cells were permeabilized with 0.1% saponin in PBS for 30 minutes followed by blocking with 10% goat serum for 1 hour. The cells were then incubated with antibodies to S100, GFAP, NGFR, and appropriate mouse and rabbit IgG antibodies overnight at 4°C. FITC and TRITC secondary antibodies were applied for 1 hour at room temperature following three washes with 0.1% saponin/ 1X PBS. The cells were then observed by confocal microscopy.

Functional co-culture assay with dorsal root ganglion cells

P1 embryonic (E16-18) dorsal root ganglion (DRG) cells (Cambrex, East Rutherford, NJ) were plated in 16-well chamber slides pre-coated with poly-D-lysine (Sigma, St. Louis, MO) at a density of 5,000 cells/well. The DRG cells were incubated for 24 hours in neurobasal media (Cambrex, East Rutherford, NJ). Twenty-four hours prior to plating the DRGs, Schwann cells, undifferentiated MSCs (uMSC) and MSCs differentiated towards a Schwann cell phenotype (dMSC) were seeded on 1μm pore size culture inserts at a seeding density of 5000 cells/insert and incubated at 37°C, 95% humidity and 5% CO_2. Forty-eight hours later, the inserts were checked for cell adherence and added to the DRG chamber

slides such that molecules secreted by the cells could permeate to the DRG cells without direct cell contact (Fig. 3-1). In addition, some wells of DRG cultures were left without co-culture with another cell type and used as controls. The co-culture was incubated at 37°C for an additional 48 hours, after which the DRG cells were fixed with 4% PFA. Fixed DRG neurons were incubated with anti-β tubulin III mouse monoclonal antibody overnight at 4°C. The following day, the neurons were incubated with AlexaFluor 488 secondary antibody for 1 hour at room temperature. The slides were examined under confocal microscopy. For each co-culture condition, 20 random fields were examined for the neurite length. The longest neurite for each DRG in the field was recorded.

Cultivation of Schwann cells

Schwann cells were harvested from adult rat sciatic nerves according to a protocol modified from Morrissey *et al* [38]. Epineural sheaths were removed from sciatic nerves and then the nerves were cut into 2mm long sections. The nerve pieces were then serially washed in Hanks Balanced Salt Solution (HBSS) three times and put into 6-well plates pre-coated with laminin (2ug/ml) and poly-D-lysine (30ug/ml). Cover slips were mounted on top of the nerve sections to ensure contact between the nerve and the plate and DMEM supplemented with 10% FBS was added. Fibroblast explantation was allowed to occur over 3-4 weeks, with media changes every 2 days. Once fibroblast migration slowed, the nerve pieces were moved to freshly coated 6 well plates and serum free Schwann cell media was added to promote Schwann cell explantation. The Schwann cell media used

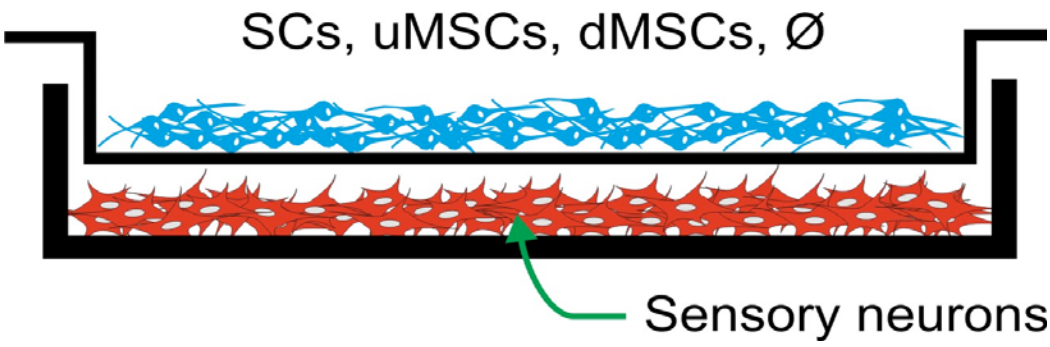


Figure 3-1: Indirect co-culture system. DRG sensory neurons were cultured on the lower culture well surface. Upper inserts were seeded with either Schwann cells, uMSCs, dMSCs or nothing. Co-cultures were left assembled for 48 hours, following which the inserts were discarded and the DRG neurons were stained with anti-tubulin βIII for quantification of neurite outgrowth.

was developed by Li *et al* and consisted of F12/DMEM supplemented with 5μg/mL insulin, 10μg/mL transferrin, 2nM progesterone, 20μg/mL BPE, 2.5nM GGF-2, 2μM Forskolin, and 5μg/mL vitamin E. Schwann cell explantation was continued for 1 month with media changes every 3 days. Explanted Schwann cells were sub-cultured using 0.25% trypsin and 1mM EDTA, and re-seeded on coated 75cm^2 plastic flasks.

In vivo transplantation of dMSCs

The effectiveness of MSC transplantation was examined using the rat sciatic nerve injury model, where a 12mm gap was bridged by a biodegradable collagen nerve guide (Integra, Plainsboro, NJ). Forty Sprague-Dawley rats, each weighing 250-275g were used. Animals were divided into 5 groups with the 12mm nerve gap bridged by an: empty nerve guide (n=8), uMSC seeded nerve guide (n=8), dMSC seeded nerve guide (n=8), Schwann cell seeded nerve guide (n=8) and autologous nerve graft (n=8) (Fig. 3-2). Anesthesia was achieved using weight appropriate doses of rompun and ketamine (7mg/kg and 75mg/kg respectively). The sciatic nerve was exposed in the right hind limb through a muscle splitting incision. A segment of the nerve was resected and the collagen nerve guide was sutured to the proximal and distal ends of the transected sciatic nerve as to create a 12mm gap. For the autologous nerve graft group, a 12mm section was resected, reversed and re-implanted. The incision was closed with a 2-layer closure. The rats were left to convalesce for 3 months, at which time EMG measurements were recorded and histological studies were performed.

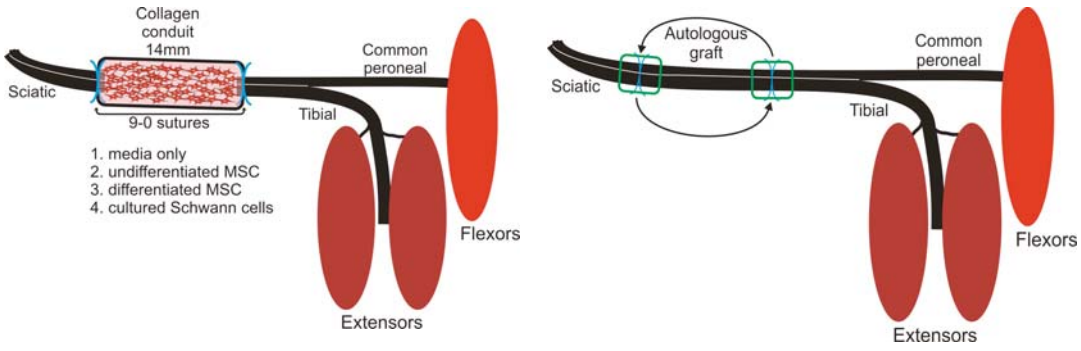


Figure 3-2: Experimental design for animal *in vivo* study. A 14mm collagen nerve guide was used to bridge a 12mm nerve gap. The nerve guide was empty or seeded with one of: Schwann cells, uMSCs, or dMSCs. A reversed autologous nerve graft was used as a positive control.

Electrophysiological studies

Electrophysiology was performed on all animals prior to sacrifice. The sciatic nerve was exposed at the sciatic notch, proximal to the graft site and an electromyographic recording needle was placed in the tibialis anterior muscle. The nerve was stimulated supramaximally with two silver wire electrodes. Compound action potentials were measured, and stimulus threshold and peak amplitude were recorded.

Back-labeling procedure using retrograde fluorescent dyes

The animals were anesthetized and the right hind limb was re-opened through the previous incision. The CP and tibial nerves were exposed and transected 10mm distal to the graft site and the proximal nerve tips were immersed in a pool of retrograde fluorescent dye, either fluororuby or fluorogold for 1 hour (Fig. 3-3). The incisions were sutured and the animals were allowed to recover from anesthesia. One week following the back-labeling procedure, the animals underwent trans-cardiac perfusion. CP and tibial nerve segments were harvested for histology. The spinal cords were harvested for counting the motoneurons that successfully regenerated axons to the site of dye application (Fig. 3-3).

Statistics

Statistical comparisons were performed using SPSS 14.0 software. Values were expressed as a mean ± standard error (SE). The non-paired student T-test

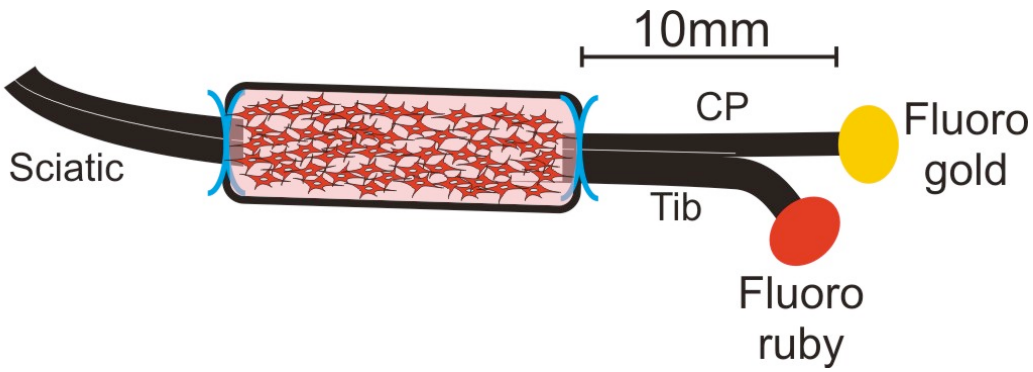


Figure 3-3: Fluorogold and fluororuby retrograde dyes were applied to the CP and tibial nerve ends cut 10mm distal to the graft site to assess the number of motoneurons regenerating axons to that site. Nerve ends were stained with the dyes for 1 hour at room temperature.

was used for comparisons of two groups or time points. When more than 2 groups were compared, ANOVA was used to determine statistical significance. Significance was accepted at 5% ($p<0.05$).

Results

MSCs obtained from bone marrow display stem cell surface marker expression and ability to differentiate into mesodermal cell lineages.

MSCs obtained from rat bone marrow were identified using fluorescence-activated cell sorting and differentiation bioassay. FACS analysis demonstrated positive expression of stem cell markers CD54 and integrin-β1 and lacked expression of hematopoietic cell markers CD14 and CD45 (Fig. 3-4). Undifferentiated MSCs had either an elongated fibroblast-like morphology or a large flat morphology (Fig. 3-5a). When cultured under appropriate culture conditions, we were able to direct differentiation of these cells into osteogenic and adipogenic phenotypes (Fig. 3-5b, c). Alizarin red, stain used to identify calcium deposits, stained positive in the MSC cultured cells directed towards an osteogenic phenotype (Fig. 3-5c). Oil red O stain, used to stain lipids, stained the lipid-laden vacuoles in the cells (Fig. 3-5b).

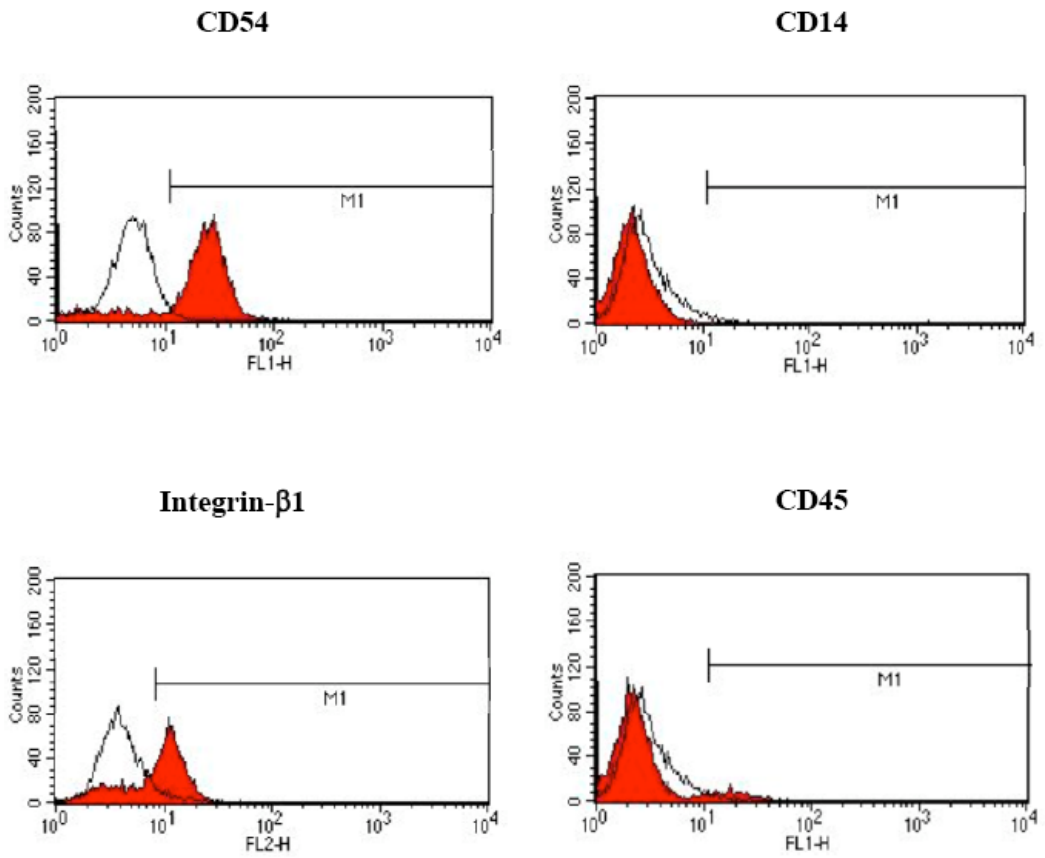


Figure 3-4: Fluorescence-activated cell sorting analysis of positive and negative MSC cell markers. MSCs expressed MSC cell markers CD54 and Integrin-β1, while demonstrating no expression of hematopoietic cell markers CD14 and CD45.

A. **B.** **C.**

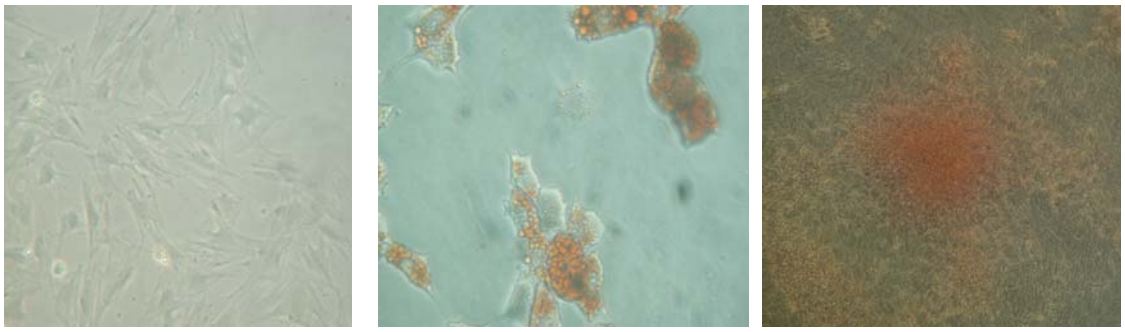


Figure 3-5: Differentiation of MSCs along mesodermal cell lineages. Undifferentiated MSCs demonstrate a large, flat, fibroblast morphology (A). Differentiation of MSCs towards an adipogenic lineage resulted in a change in morphology and development of lipid-laden vacuoles, which stained positive for Oil red O (B). Differentiation of MSCs along an osteogenic lineage resulted in the production of calcium matrix which stained positive for Alizarin red (C).

A. **B.** **C.**

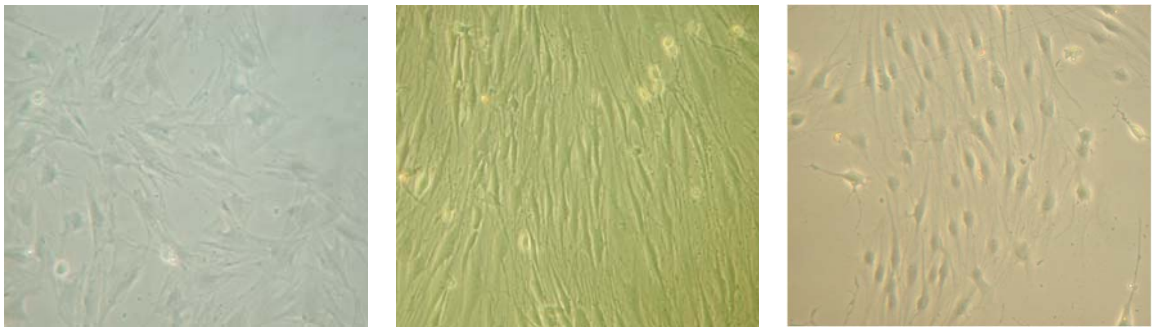


Figure 3-6: With differentiation of MSCs towards a Schwann cell phenotype, the cell went form large flat fibroblast-like cells (A) to slender, elongated cells with terminal processes (B) similar to that seen in cultured Schwann cells (C). Additionally, the pattern of growth became linear and parallel similar to Schwann cells.

MSCs differentiated into Schwann like cells with expression of Schwann cell markers.

MSCs were directed towards a Schwann cell phenotype using defined culture medium. Changes in cell morphology were observed initially with the addition of β-mercaptoethanol and retinoic acid, where the large flat shaped MSCs became more slender. With the addition of the growth factor transdifferentiation media, the cells progressively became more elongated and slender, similar in morphology to Schwann cells (Fig. 3-6). Additionally, undifferentiated MSCs grew in a random pattern, overlapping and clustering where as with differentiation, the cells proliferated in a longitudinal, parallel manner, similar to that observed in cultured Schwann cells.

The expression of Schwann cell markers by these differentiated MSCs (dMSCs) was evaluated using immunocytochemistry and fluorescence-activated cell sorting at different time points of treatment. Differentiated cells stained positive for Schwann cell markers S100, GFAP and NGFR at days 2, 6, 10, and 14, while the undifferentiated MSCs (uMSC) failed to demonstrate positive staining for any of the three markers (Fig. 3-7). Schwann cell marker expression was quantified using flow cytometry. MSC differentiation into Schwann like cells was a heterogeneous process with 51% of cells expressing GFAP, 47% of cells expressing S100 and 45% of cells expressing NGFR after 6 days of treatment with transdifferentiation medium (Fig. 3-8g,h,i). In comparison, the uMSCs demonstrated no expression of Schwann cell markers (Fig. 3-8d,e,f).

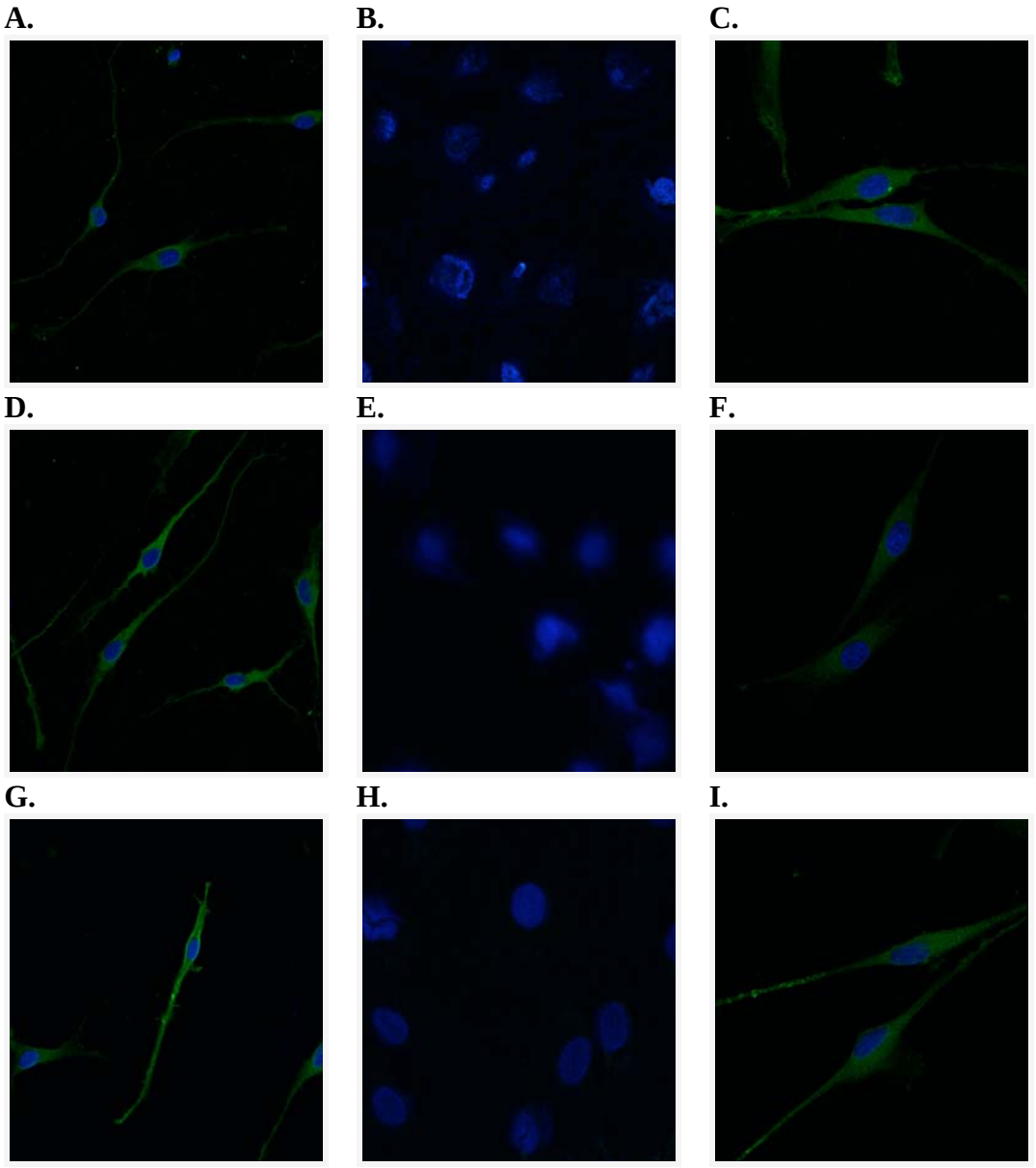


Figure 3-7: Immunocytochemical staining for Schwann cell markers. Schwann cells stained positive for GFAP (A), S100 (D) and NGFR (G). Undifferentiated MSCs did not stain positively for GFAP, S100 or NGFR (B, E, H respectively). MSCs differentiated towards a Schwann cell phenotype, like Schwann cells, stained positive for all three markers, GFAP (C), S100 (F) and NGFR (I). Cells were observed using confocal microscopy.

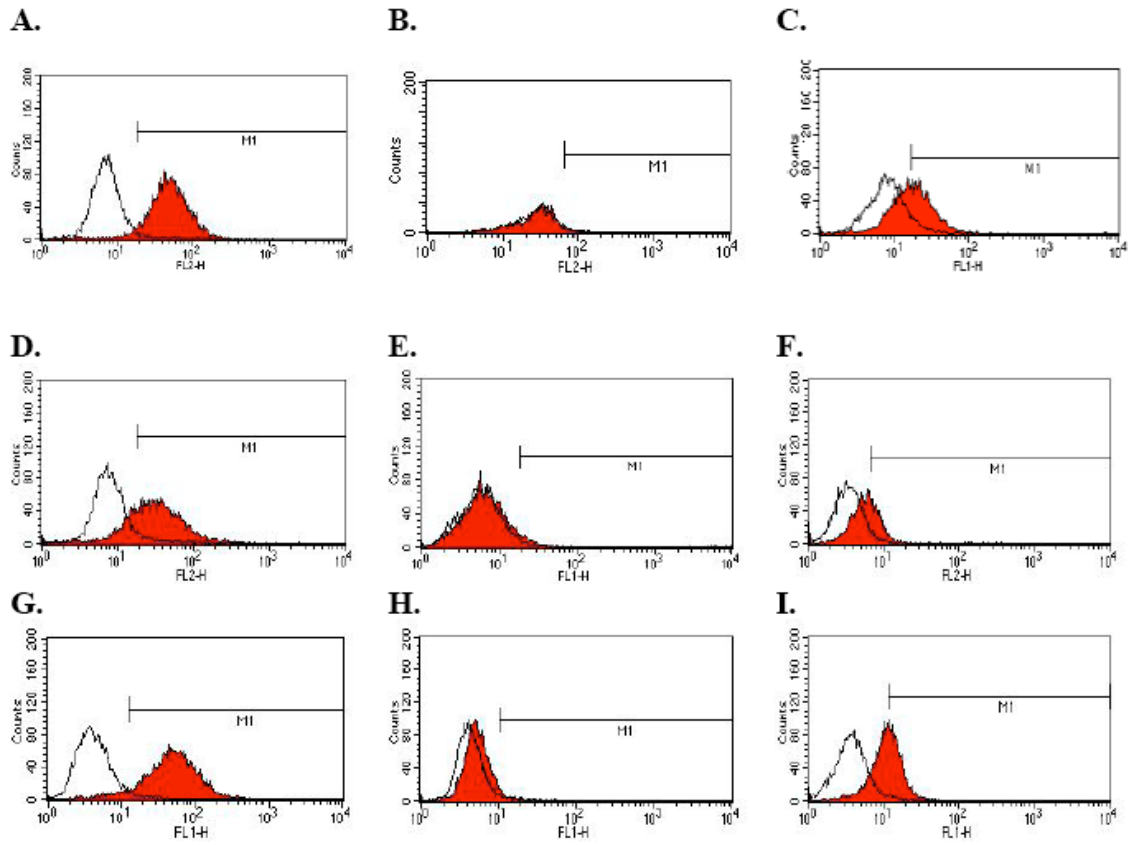


Figure 3-8: FACS analysis of Schwann cell marker expression. Schwann cells markers demonstrated strong expression of GFAP, S100 and NGFR (A, D, G) serving as a positive control for antibodies used. Undifferentiated MSCs fails to express GFAP, S100 and NGFR (B, E, H). Approximately 50% of dMSCs expressed Schwann cell markers GFAP, S100, and NGFR (C, F, I).

Thus, the differentiation process was heterogeneous with approximately 50% of cells present in culture differentiating along a glial lineage. Schwann cell marker expression was examined over time. Maximum maker expression was observed after 6 days of treatment with transdifferentiation medium after which marker expression decreased. There was a significant increase in Schwann cell marker expression from day 2 to day 6 and a significant decrease in marker expression from day 6 to day 14 for all 3 markers ($p<0.05$) (Fig. 3-9). Thus, differentiation of MSCs into a Schwann cell phenotype with expression of Schwann cell markers is transient *in vitro* with maximum marker expression after 6 day of treatment with transdifferentiation media.

MSCs differentiated towards a Schwann cell phenotype exert similar neurotrophic effects as Schwann cells *in vitro*.

Functional properties of dMSCs were evaluated *in vitro* using an indirect co-culture bioassay. On examination of the stained DRG neurons under confocal microscopy, there was a noticeable difference in neurite extension between the groups, with the Schwann cell and dMSC co-culture groups resulting in longer and more arborous neurite outgrowth (Fig. 3-10). Co-cultures of DRG neurons with Schwann cells resulted in the longest neurite outgrowth (0.32±0.019mm) followed by dMSC co-cultures (0.30±0.018mm), and uMSC co-cultures (0.22±0.015mm) (Fig. 3-11). DRG neurons cultured alone resulted in a mean neurite length of 0.13±0.022mm. The mean neurite length of the Schwann cell, dMSC and uMSC co-culture groups were significantly greater than the DRG

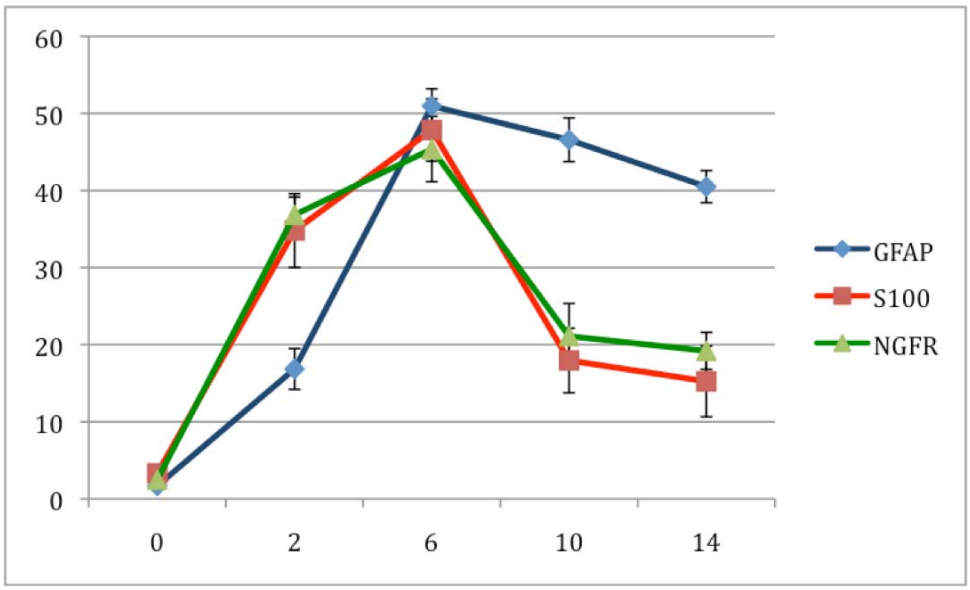


Figure 3-9: Temporal expression of Schwann cell markers. At day 0, MSCs demonstrated no expression of Schwann cell markers. Marker expression was observed at day 2 of growth factor treatment and maximum expression of GFAP, S100 and NGFR was observed at 6 days of treatment with growth factors. Expression levels decreased with time thereafter at days 10 and 14.

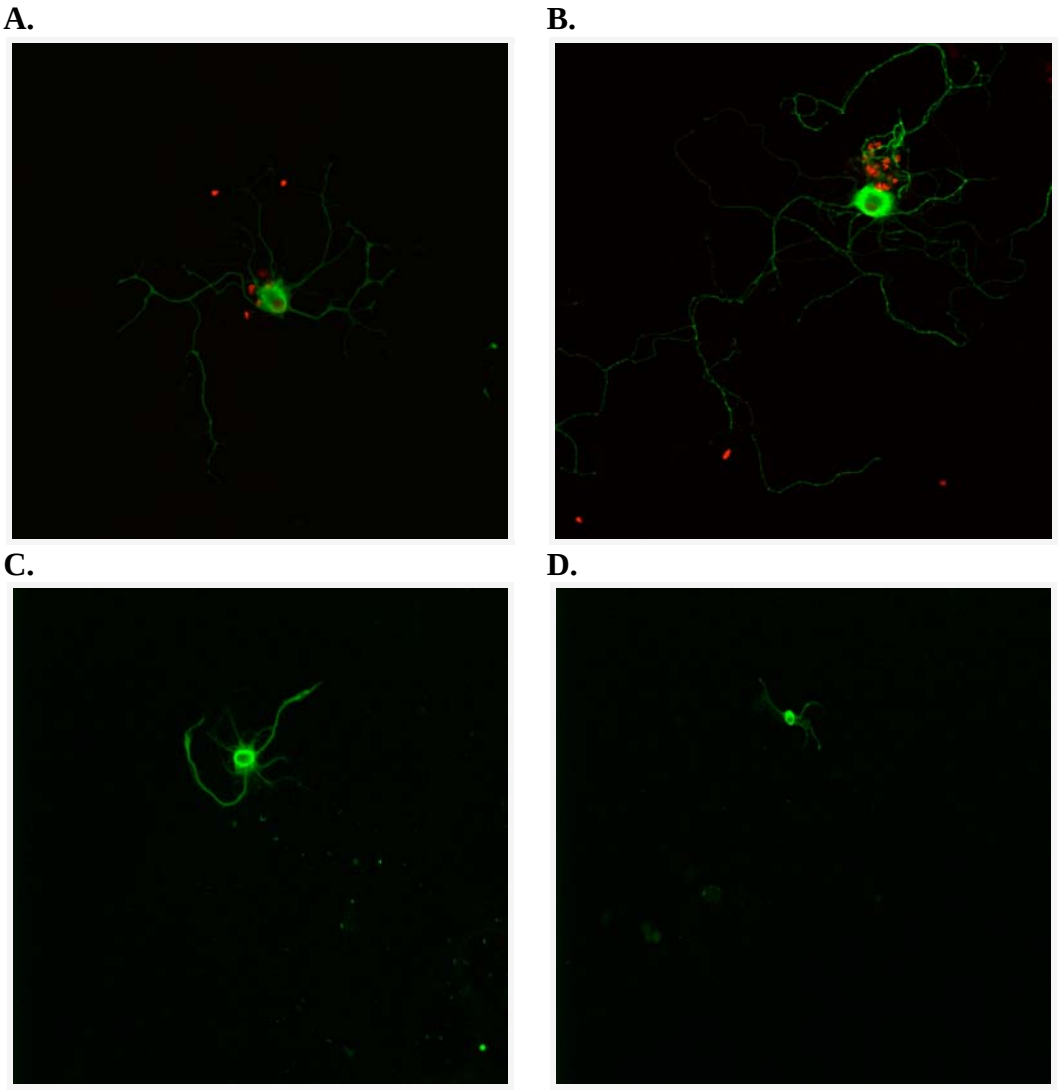


Figure 3-10: DRG cells stained with anti-tubulin βIII following a 48 hour co-culture with (A) Schwann cells; (B) dMSCs; or (C) uMSCs. DRG cells cultured alone resulted in minimal neurite extension in 48 hours (D).

alone culture group ($p<0.05$). Comparing between the groups, there was no statistically significant difference in mean neurite length between the Schwann cell group and the dMSC group, indicating equivalent neurotrophic effect. The neurite lengths in both the Schwann cell and dMSC groups were statistically greater than the uMSC group ($p<0.05$).

MSCs differentiated along glial lineage promote axon outgrowth *in vivo.*

After 3 months regeneration, the animals were analyzed for functional and histological evidence of axon regeneration. During the course of study there was some degree of self-mutilation of the denervated limb in a few of the animals, but not severe enough to remove them from the study. On re-opening the wounds, the collagen conduit was still present, although much thinner. There was minimal adhesion to surrounding tissues and the nerve guide appeared to be well vascularized with numerous blood vessels coursing along the surface.

The capacity of dMSCs to promote axonal outgrowth *in vivo* was evaluated by counting the number of motoneurons that were back-labeled with fluorescent dyes. The number of motoneurons regenerating axons across the nerve gap was highest in the autograft group (1332.33±30.48) and was statistically significantly higher than all other experimental groups ($p<0.05$). The number of motoneurons regenerating axons across the nerve gap in the Schwann cell and dMSC seeded nerve guide groups were 817±91.8 and 627.75±86.0

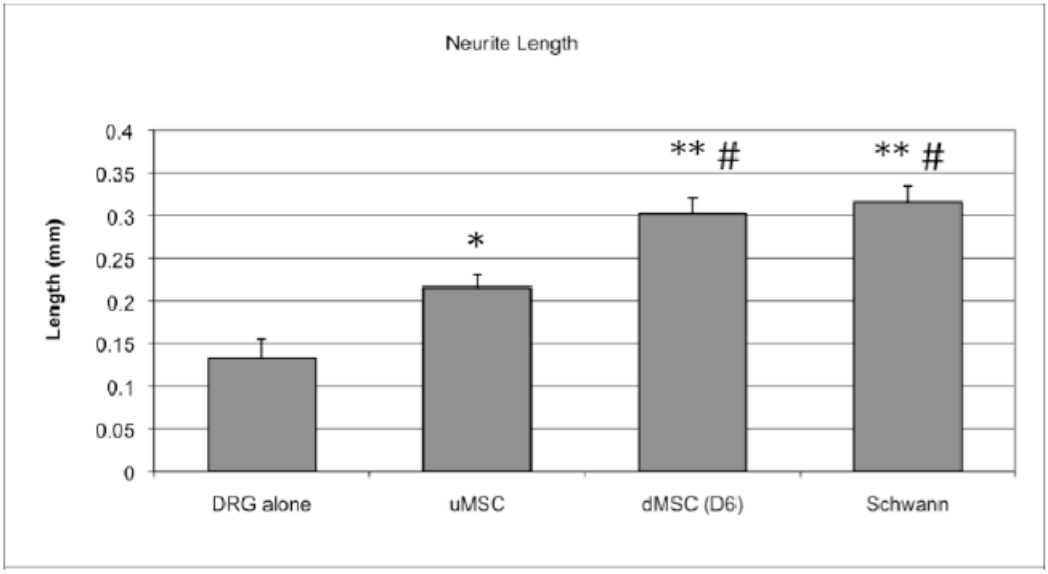


Figure 3-11: The mean length of the longest neurite extending from each DRG. Lengths were longest in the Schwann cell co-culture and dMSC groups, which were significantly greater than the uMSC and DRG alone group. The neurite length in the uMSC group was significantly greater than the DRG alone group. (*$p<0.05$ compared to DRG alone, **$p<0.001$ compared to DRG alone, #$p<0.05$ compared to uMSC group)

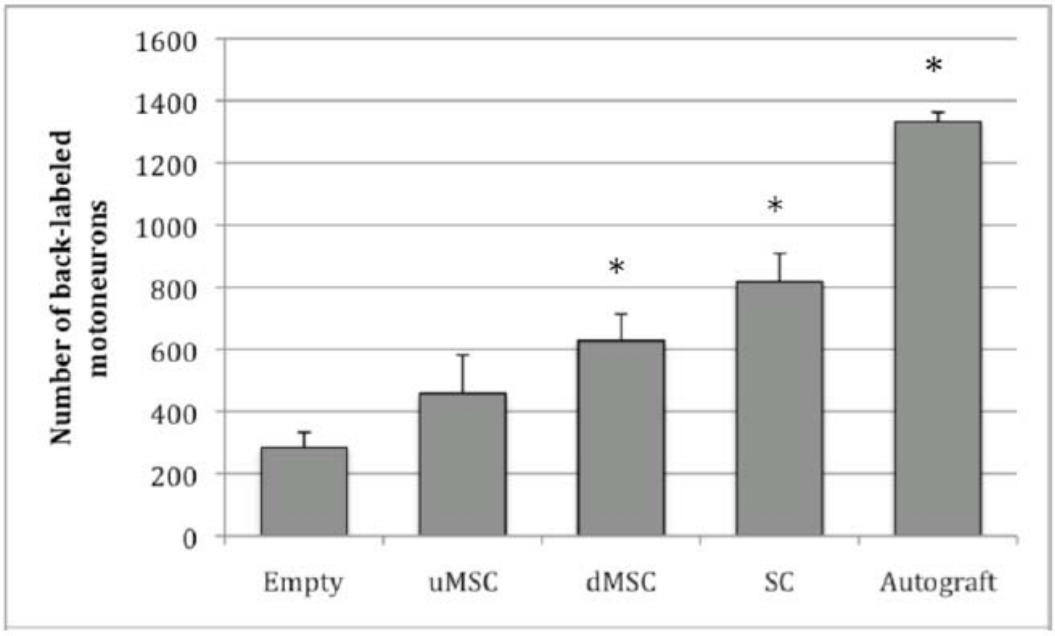
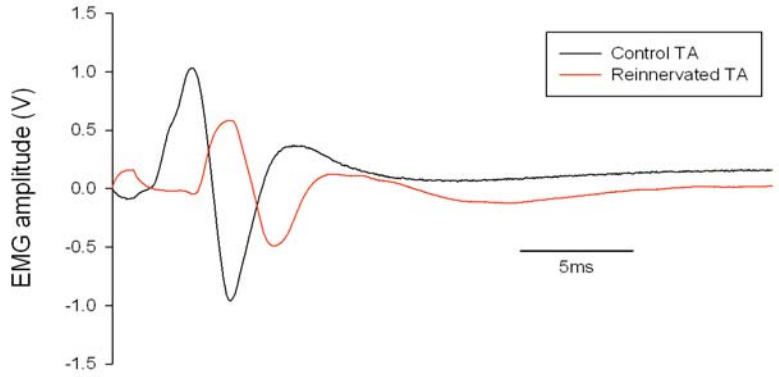


Figure 3-12: There was a significant increase in the number of motoneurons (MNs) regenerating axons across the nerve gap with the use of nerve guides seeded with Schwann cells and dMSCs compared to an acellular nerve guide. The use of an autologous nerve graft demonstrated the highest number of MNs regenerating axons across the nerve gap. The number of MNs regenerating axons was not significantly higher in nerve guides seeded with uMSCs compared to an acellular nerve guide (*$p<0.05$). N=8 in each group.

respectively (Fig. 3-12). There was no statistically significant difference between the two groups and both were significantly greater than the number of motoneurons regenerating axons in the empty nerve guide group, the lowest number of motoneurons regenerating their axons (284.83±48.1) ($p<0.05$). Regeneration of axons by motoneurons in the uMSC group was not significantly greater than the empty conduit group (Fig. 3-12).

Functional reinnervation was assessed by electrophysiology and muscle weights. EMG measurements were recorded at the tibialis anterior (TA) muscle. EMGs obtained from reinnervated TA muscles were approximately 50% of normal peak size (Fig. 3-13a). There was no significant difference in peak-to-peak EMG measurements between any of the experimental groups, which were all significantly lower than a normal intact TA muscle (Fig. 3-13b). Corresponding normalized TA muscle weights (ratio experimental right limb to normal left hind limb) demonstrated a similar trend with no significant difference in any of the cell seeded conduit groups, which were all significantly lower than the autograft group (Fig. 3-14).

A.



B.

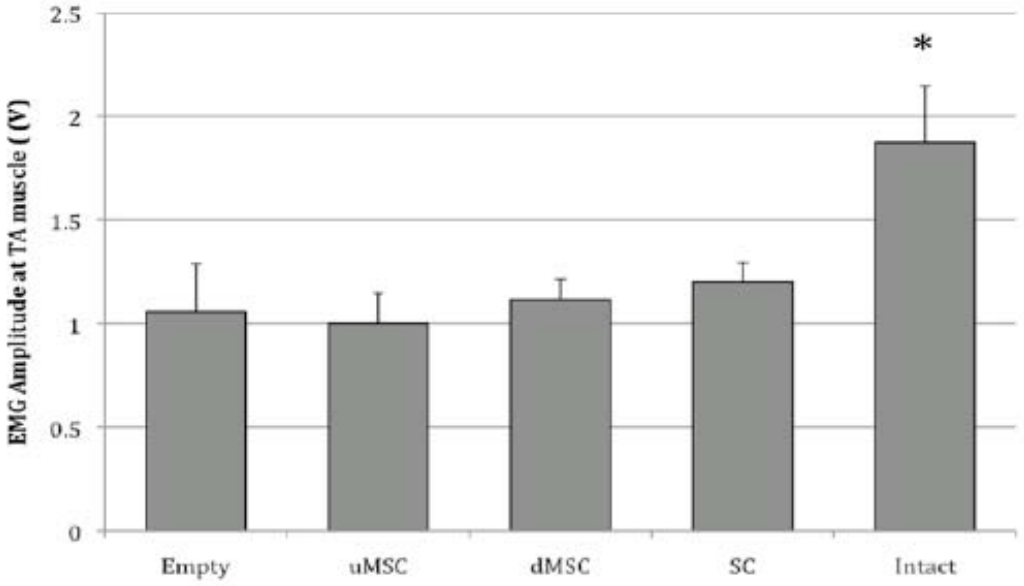


Figure 3-13: EMG amplitude measurements were recorded at the tibialis anterior muscle. Re-innervated TA muscle demonstrated an EMG recording significantly decreased from intact TA muscle (A). No differences in EMG measurements were observed between the experimental groups, which were all significantly lower than the EMG of an intact TA muscle (B) (*$p<0.05$).

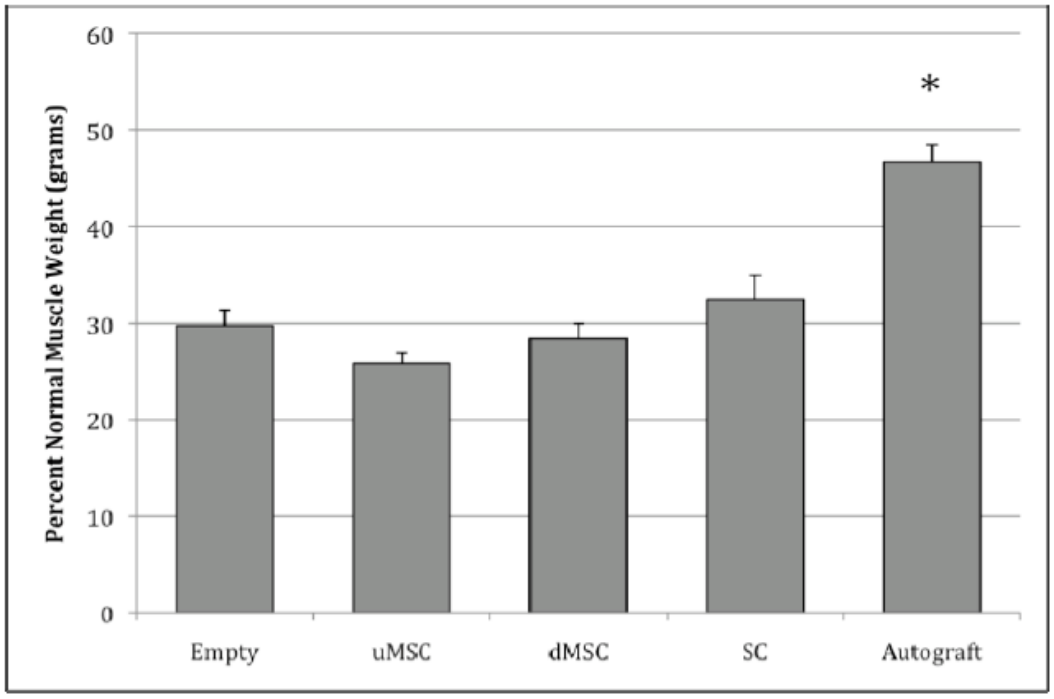


Figure 3-14: The muscle weight of the tibialis anterior muscle of the experimental right hind limb was expressed as a ratio to the normal left side. Tibialis muscle weights were not significantly different in any of the nerve guides seeded with cells compared to the acellular nerve guide. The autograft group demonstrated increased tibialis anterior muscle weights compared to the other groups (*$p<0.05$).

Discussion

Peripheral nerve injuries resulting in nerve gaps present a challenging reconstructive problem. Restoration of nerve continuity is essential for axon regeneration and the current standard of bridging nerve gaps is autologous nerve grafting. Autologous nerve graft represents the ideal conduit to bridge a nerve graft as it consists of a basal lamina conducive to Schwann cell and axon migration, and it contains viable Schwann cells which facilitate axon regeneration by forming Bands of Büngner in the distal nerve stump[16,48] and secreting neurotrophic factors[9,17,33]. The importance of Schwann cells in peripheral nerve regeneration has been long recognized, where regeneration across nerve gaps in acellular conduits is greatly limited across gaps greater than 2cm[22,24,25]. Although ideal, the use of autologous nerve grafts and engrafted exogenous Schwann cells are limited by the requirement of a donor nerve leading to donor site deficits in function. Additionally, Schwann cells have limited ability to expand *in vitro* further limiting their use in clinical situations. Due to these limitations, alternate methods of bridging nerve gaps have been explored.

Bone marrow derived mesenchymal stem cells have recently demonstrated the ability to differentiate along neuronal and glial lineages[1,10,43,51], and thus have been the focus of recent investigation as a cellular adjunct to alternative nerve guides. MSCs isolated from the bone marrow have been identified through pluripotency along mesodermal cell lineages and more recently by a panel of cell surface markers, which has allowed for more accurate identification and

characterization of MSCs[41]. In this study, MSCs were isolated from the bone marrow and characterized based on cell surface markers and differentiation bioassay. MSC cultures appeared heterogeneous under light microscopy. As expected, the MSCs isolated from the rat bone marrow lacked expression of hematopoietic cell makers CD14 and CD45. Approximately 70% of cells present in culture expressed CD54 and Integrin-β1 indicating the presence of accessory cells including macrophages, fibroblasts and endothelial cells[6,28]. The role of these accessory cells on MSC pluripotency and self-renewal remains undefined[27]. As described in the literature, under defined culture conditions, these cells were able to assume osteogenic and adipogenic phenotypes, thereby confirming pluripotency[41,42].

The ability of MSCs to differentiate along a Schwann cell lineage was first described by Dezawa *et. al.* [12]. Other studies have repeated this differentiation process with slight modifications[29,31,49] and characterized the differentiated MSCs using qualitative or semi-quantitative methods. In this study, Schwann cell marker expression and permanence of the Schwann cell phenotype over time was characterized *in vitro*. Differentiation of MSCs into a Schwann cell phenotype was achieved through sequential induction with β-mercaptoethanol and retinoic acid, followed by treatment with FGF, PDGF, forskolin and GGF2. β-mercaptoethanol (BME), a reducing agent, has been shown to induce morphological cellular changes and when added to MSCs, has been shown to induce the formation of nerve cells[10,11,51]. Retinoic acid (RA) has been shown to

induce differentiation of embryonic stem cells and neuronal progenitors into nerve cells when used in combination with NGF[43]. Thus, the use of β-mercaptoethanol and retinoic acid served to prime the MSCs towards a neuronal phenotype.

Following induction of MSCs with BME and RA, the cells were treated with a combination of growth factors. GGF2, a member of the neuregulin family, has been shown to direct cell lineage of neural crest cells towards Schwann cells[45]. Additionally, neuregulins are trophic for Schwann cell precursors and at higher concentrations drive proliferation and maturation[14]. FGF has been shown to potentiate the effects of neuregulins in Schwann cell development[15,52]. PDGF has been implicated in preventing Schwann cell apoptosis and promoting Schwann cell survival. Forskolin up-regulates cAMP and thus serves as a mitogen, promoting Schwann cell proliferation. All of these factors work synergistically on MSCs to direct differentiation towards a Schwann cell phenotype as omission of any single factor disrupts the differentiation process[12].

Here, we demonstrate that MSCs are capable of expressing Schwann cell markers, including S100, GFAP and NGFR. As described by other studies, the dMSCs stained positive for Schwann cell markers on immunocytochemical staining[12,29,31,37]. These studies described MSC differentiation in qualitative terms, with some studies attempting to describe the differentiation process semi-quantitatively. Although claiming that MSCs can express Schwann cell markers, the actual percentage of cells differentiating into Schwann like cells was not

addressed. In this study, we found that approximately 50% of cells present in a MSC culture passaged to P2 differentiate along a glial lineage and express Schwann cell markers. In comparison to a study by Lin *et al* where 50% of MSCs differentiated towards a Schwann like phenotype expressed S100, 36% expressed GFAP and 32% expressed NGFR, our differentiation protocol yielded slightly better Schwann cell marker expression[34]. The main difference in differentiation between the two studies is that our protocol utilized GGF-2 as the neuregulin; whereas Lin *et. al.*[34] utilized heregulin as originally described by Dezewa *et. al.*[12] The heterogeneous nature of MSC differentiation can be attributed to a number of factors. First, as discussed previously, MSC cultures are heterogeneous, with only a certain proportion of the cell culture consisting of true pluripotent stem cells[13,27]. Additionally, MSCs differ from embryonic stem cells in that they demonstrate senescence and limited capacity for differentiation with time[13]. This heterogeneity greatly limits standardization of cultures and differentiation protocols, having significant implications for tissue engineering and future clinical application of these cells.

Schwann cell marker expression of dMSCs varied greatly with time. Schwann cell marker expression increased from very little in the uMSCs to a maximum level of expression seen at 6 days of treatment with growth factors. Interestingly, longer treatments with the growth factor cocktail resulted in a decline in Schwann cell marker expression. These findings are in agreement with Lin *et. al.*, where maximum expression of S100 was found to be after 6 days of

treatment with the transdifferentiation medium[34]. Expression of the other markers was not evaluated their study. The mechanism behind this decline in differentiated phenotype is not known, but might be attributed to cell senescence and apoptosis. In addition, this marker expression profile is under specific *in vitro* conditions and may be altered by different culture conditions or *in vivo*. Keilhoff *et. al.* demonstrated that if MSCs differentiated into Schwann like cells were cultured in basic growth medium, the Schwann cell phenotype was lost by 3 days; however these de-differentiated cells were capable of re-differentiating with the appropriate culture media[31]. Permanence of differentiation in MSCs requires further investigation before being considered for clinical application.

In addition to Schwann cell marker expression, a functional bioassay was performed to evaluate neurotrophic properties of MSCs differentiated towards a Schwann cell phenotype. Differentiated MSCs demonstrated equivalent positive effect on DRG cell neurite outgrowth as Schwann cells, thereby providing *in vitro* evidence for the ability of dMSCs to secrete neurotrophic factors. It has been shown that dMSCs secrete BDNF and NGF when co-cultured with DRG sensory neurons[36] and thus function like Schwann cells in promoting neurite outgrowth. As with Schwann cell marker expression, the permanence of this functional capacity has yet to be characterized. There have been some reports in the literature as to the potential of undifferentiated MSCs to exert neurotrophic effects when transplanted into a nerve gap *in vivo*[5,7]. Our bioassay results demonstrate that uMSCs have minimal neurotrophic effect which is insufficient to explain the

improved axonal regeneration found in these studies, indicating that MSCs possibly function along other pathways to facilitate axonal regeneration.

In vivo, we found that transplantation of dMSCs into a collagen nerve guide used to bridge a 12mm nerve gap resulted in improved axonal regeneration across the gap, similar to that of transplanted Schwann cells. Similar results were found in other studies where dMSCs resulted in improved regeneration histologically[29,31,32,49]. These results also correspond with what was predicted from our *in vitro* co-culture bioassay. Transplantation of uMSCs, however, failed to demonstrate any increase in the number of motoneurons regenerating axons across the nerve gap when compared to the empty nerve guide. This finding is consistent with the results of the *in vitro* bioassay and with some studies in the literature, where uMSC transplantation yielded regeneration equivalent to an empty tube[31,32]. However, other studies have demonstrated improved axon regeneration across a nerve gap with transplantation of non-differentiated MSCs[5,7,8]. Based on our *in vitro* characterization of MSCs, one would not expect a significant neurotrophic effect from uMSCs *in vivo*. Chen *et al*[5] demonstrated that uMSCs can secrete BDNF and NGF and thus can function to promote axonal regeneration, however this was in direct contrast to a study by Mahay *et. al.*[36], who reported little to no production of BDNF and NGF. MSCs are extremely sensitive to culture conditions including culture medium and substrate and variations in these conditions could, in part, explain discrepancies in the literature[27].

Electrophysiology and muscle weight were used as functional outcome measures in this study. We observed no improvement in functional outcome measures with transplantation of any cell type over an acellular nerve guide. EMG analysis demonstrated equivalent peak-to-peak values for all experimental groups, all of which were significantly lower than normal. Similarly, we observed no significant difference in muscle weights in any of the experimental groups, except the autograft group, which was significantly greater than the other groups. The lack of functional improvement observed can be attributed to misdirection of reinnervation and regenerative axonal sprouting at the neuromuscular junction. Animal studies have demonstrated that following nerve transection injury, regenerating axons have no predilection to reinnervate their original muscle[18,19,47]. The sciatic nerve is a large poly-fascicular nerve with two main branches, the CP and tibial nerves. With injury and repair of the sciatic nerve, regenerating axons have equal probability of regenerating into either the CP or tibial branches, potentially causing alterations in reinnervation patterns. In addition to misdirection, it has been demonstrated that with partial dennervation of a muscle, terminal axonal sprouting occurs to compensate for a decreased number of motoneurons innervating the muscle[26,46]. In our study, the number of motoneurons that regenerated across the nerve gap to muscle was significantly lower than an intact nerve. This partial reinnervation resulted in terminal axonal sprouting whereby motor unit sizes increased to reinnervate all the muscle fibers in the muscle. In such a scenario, no difference in peak-to-peak EMG

measurements would be expected. Similarly with muscle weights, terminal sprouting would compensate for a decreased number of axons regenerating to muscle, resulting in equivalent reinnervation of the tibialis muscle. The autograft group did demonstrate an increase in tibialis muscle weight. The use of an autograft resulted in significantly higher numbers of motoneurons regenerating across the nerve gap and thus the degree of terminal sprouting at the muscle was less. This could lead to quicker reinnervation and activity of muscle fibers, thereby maintaining muscle size.

Reinnervation across a nerve gap constitutes a complicated clinical problem. Autologous nerve grafting remains the standard of care however requires sacrifice of a normal nerve. Recent evidence has demonstrated the efficacy of enhancing alternative nerve guides with Schwann cells to bridge large nerve gaps; however, this also requires the sacrifice of a normal nerve. MSCs present an endless supply of rapidly proliferating cells for use in tissue engineering. The use of MSCs in peripheral nerve repair has led to some promising results. In this study, we demonstrated the ability of these cells to differentiate into and function as Schwann cells both *in vitro and in vivo*. Despite these observations, there remain many uncertainties that require further investigation before MSCs can be translated to clinical application including heterogeneity of MSC culture, incomplete differentiation to a desired phenotype and permanence of the differentiated phenotype. These will be the focus of future study.

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Chapter 4: Rolipram-induced elevation of cAMP or chondroitinase ABC breakdown of inhibitory proteoglycans in the extracellular matrix promotes peripheral nerve regeneration*

Abstract

The inhibitory growth environment of myelin and extracellular matrix proteoglycans in the central nervous system may be overcome by elevating neuronal cAMP or degrading inhibitory proteoglycans with chondroitinase ABC (ChABC). In this study, we asked whether similar mechanisms operate in peripheral nerve regeneration where Wallerian degeneration removes myelin and extracellular proteoglycans slowly. We repaired transected common peroneal (CP) nerve in rats and either elevated cAMP in the axotomized neurons by subcutaneous rolipram, a specific inhibitor of phosphodiesterase IV, and/or promoted degradation of proteoglycans in the distal nerve stump by local ChABC administration. Rolipram treatment significantly increased the number of motoneurons that regenerated axons across the repair site at one and two weeks, and increased the number of sensory neurons that regenerated axons across the repair site at two weeks. Local application of ChABC had a similar effect to rolipram treatment in promoting motor axon regeneration, the effect being no greater when rolipram and ChABC were administered simultaneously. We conclude that blocking inhibitors of axon regeneration by elevating cAMP or degrading proteoglycans in the distal nerve stump promotes peripheral axon regeneration after surgical repair of a transected nerve. It is likely that elevated

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cAMP is sufficient to encourage axon outgrowth despite the inhibitory growth environment such that simultaneous enzymatic proteoglycan degradation does not promote more axon regeneration than either elevated cAMP or proteoglycan degradation alone.

Introduction

Axons in the peripheral (PNS) but not the central nervous system (CNS) regenerate after injury[20-22]. Nevertheless there is only a short window of opportunity for effective PNS regeneration to occur, whereby regenerative success of injured nerves progressively decreases after prolonged axotomy and Schwann cell denervation[20,21,27,52]**.** Axon outgrowth from the proximal stump of transected and surgically repaired nerves is a slow process[9] and this lengthy period when regenerating axons wander in the suture site of surgically repaired peripheral nerves[12,57] accounts, at least in part, for the delays of several weeks for all regenerating axons to cross a repair site[9]. Chondroitin sulfate proteoglycans (CSPGs) of the extracellular matrix and myelin-associated inhibitors, both potent inhibitors of neuronal regeneration in the CNS[42,49,54], are also present in the peripheral nerve. These molecules are upregulated after nerve injury, show neurite-inhibitory activity[7,50,58], and may play a role in delayed axon outgrowth following peripheral nerve injury. The prolonged time course of weeks for effective removal of myelin debris by macrophages and Schwann cells[4,17,24,51] and for degradation of glycoproteins of the extracellular matrix[33] may account for the staggered outgrowth of axons from the proximal stump of an injured nerve[3,27].

Injured nerves in the CNS may be stimulated to regenerate their axons despite the inhibitory environment by interfering with signaling pathways that are activated by inhibitory myelin associated molecules[11,15,37,43,44,47]. The inhibition can be overcome by increasing neuronal cAMP levels *in vivo*[11,15,37,43,44,47] and *in*

vitro[11] and by selectively cleaving glycosaminoglycan (GAG) side chains from the protein core of proteoglycans with chondroitinase ABC (ChABC)[18]. However, applying these pharmacological approaches to PNS regeneration has generated conflicting data. Some have reported that cAMP does promote axon outgrowth [26,35,46] while others have failed to demonstrate increased axon outgrowth[6,31,40]. The use of ChABC in the PNS has been shown to accelerate axon outgrowth into distal nerve stumps and acellular nerve grafts in rats[36,59]. Analyses of the distance of regenerating axons of *thy-1 YFP-H* transgenic mice demonstrated longer axon profiles in nerve grafts from wild-type littermates with ChABC, which specifically degrades CSPGs but not other proteoglycans such as heparin sulfate proteoglycans[30].

Using retrograde labeling techniques, we aim to further investigate whether elevation of cAMP or removal of GAG side chains from inhibitory proteoglycans in axotomized motor and sensory neurons in the peripheral nerve promotes PNS regeneration. We also question whether a combinational strategy of rolipram and ChABC that has proven to be effective in CNS regeneration[19,32,55], is also effective in the PNS.

Methods

All experiments were performed on adult female Sprague Dawley rats (200-220g) and approved by local authorities (Health Sciences Laboratory Animal

Services, University of Alberta) according to the Canadian Council for Animal Care guidelines.

Surgery: Nerve repair and delivery of rolipram and/or chondroitinase ABC (ChABC)

All surgeries were performed under Ketamine (Vetalar, Bioniche, Belleville, Ontario) and Xylazine (Rompun, Bayer, Toronto, Ontario) anesthesia at doses of 0.6 and 0.4 mg/kg intraperitoneally respectively. Eye lubricant (Duratears Naturale, Alcon, Ontario) was used to prevent corneal damage during surgery.

Using aseptic technique, the right CP nerve was exposed distal to the sciatic notch and transected 10 mm proximal to its entrance into the flexor muscle group including the tibialis anterior muscle (Fig 4-1A). Silicone silastic guides (Helix Medical, Inc, Carpinteria, CA) of 0.76mm internal diameter and 3mm length were implanted, and the proximal and distal CP nerve stumps were approximated within the nerve guide using 9-0 Ethicon nylon suture (Ethicon, Inc Somerville, NJ, USA) as described in Furey *et. al.* [23]. An Alzet mini-osmotic pump (Durect Corporation, Cupertino, CA) was then implanted subcutaneously on the back of the rat to release its content of either rolipram or saline systemically. The Alzet pump (model 2ML2**)** was filled with the appropriate solution for 24 hours and incubated at 37ºC prior to implantation. The pump delivered either rolipram[45] diluted in 1:1 Saline/Dimethyl sulfoxide (DMSO)

(rolipram group, n=26) or vehicle solution (1:1 Saline/DMSO; control group, n=22), both delivered at a rate of 0.4μmol/kg/h continuously over 7, 14 or 21 days. The surgical wound was sutured closed and the rats were monitored and kept warm until they recovered from the anesthetic.

Chondroitinase ABC application

In 2 groups of rats, the CP nerve was exposed and transected under surgical anesthesia 10mm proximal to the entrance of the nerve into the flexor muscle group of the hind limb. In the first group of animals, 5mm of the cut end of the distal stump was dipped into a Vaseline well containing 20U/ml protease free ChABC (Seikagaku, Japan) in 1% protease free bovine serum albumin for 1h. The nerve ends were then repaired using a silicone silastic guide. An Alzet mini-osmotic pump was then placed on the back of the animal and a vehicle solution of 1:1 Saline/DMSO was delivered systemically over 2 weeks at a delivery rate of 5μl/ml (ChABC group n=9). In the second group of rats, the ChABC was again applied to the distal nerve stump as described, and an Alzet pump implanted on the back of the rat was filled with rolipram solution for systemic delivery over a 2 week period at the same rate as for the vehicle solution group (Roli+ChABC group, n=8). The skin was closed after surgery and the rats were warmed and monitored during recovery from the anesthetic.

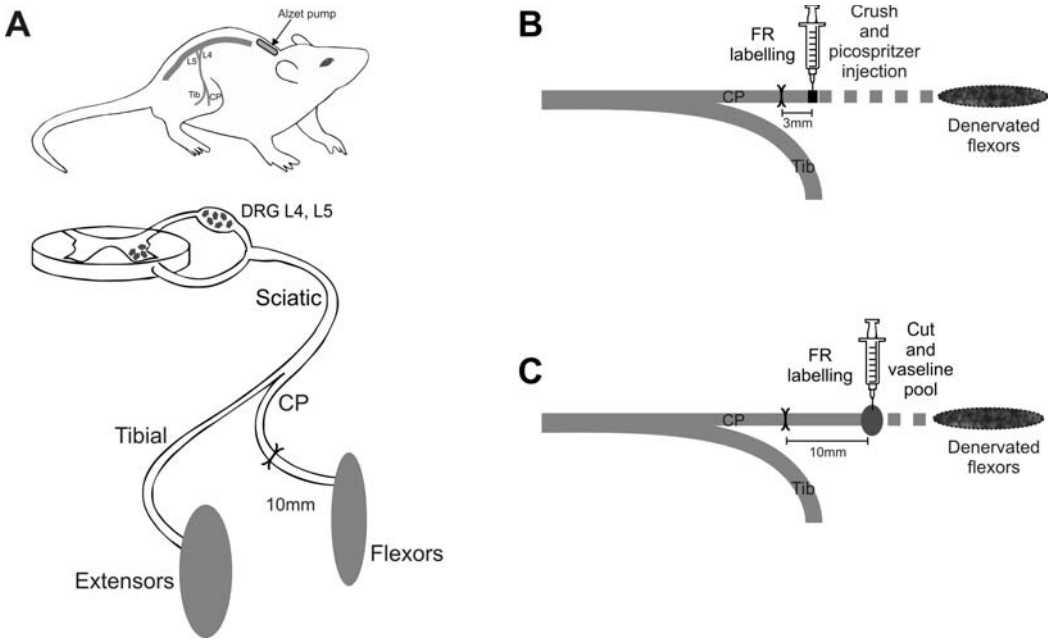

Figure 4-1: The *in vivo* model of peripheral nerve regeneration in the common peroneal (CP) nerve in the rat. A. The CP nerve was exposed, transected 10 mm proximal to the nerve entry into the flexor group of muscles that includes the tibialis anterior muscle, and repaired by tubulization. The Alzet osmotic pump was implanted subcutaneously at the back of the rats after the nerve surgery. B. After 7 or 14 days, the nerve was re-exposed, crushed 3mm distal to the suture site and fluororuby was injected into the nerve via a micropipette that was attached to a picospritzer. C. After 14 or 21 days, the nerve was re-exposed, cut 10mm distal to the suture site and the proximal stump was dipped for 1h in a solution with fluororuby contained in a Vaseline pool. Details are provided in the text.

Surgical application of retrograde dyes

A second sterile surgery was carried out under surgical anesthesia at 7 to 21 days following the CP nerve section and repair, and the systemic delivery of saline/DMSO vehicle or rolipram, and/or local application of ChABC to the CP nerve suture site. Fluororuby (Dextran, tetramethylrodamine, Invitrogen, Molecular Probes, Eugene, Oregon) was either injected 3mm distal to the site of the nerve repair or applied directly to the proximal stump of the CP nerve 10 mm distal to the repair site (Fig. 1B,C). For the microinjection, the CP nerve was crushed just distal to the cuff (3mm distal to the suture site) and the dye was injected via a micropipette that was attached to a picospritzer (Intercel Picospritzer III) [9]. For the application of the dye to the proximal stump of the CP nerve cut 10 mm distal to the suture site, the stump was dipped in 5% Fluororuby solution contained within a Vaseline well for 1 hour. Following the dye exposure, excess dye was carefully rinsed off before suturing the skin and allowing the rats to wake. In control experiments, we injected the dye at 3mm distal to the suture site immediately after nerve repair rather than after 7, 14, and 21 days. This was done to ensure that the injected dye was confined to the injection site within the distal nerve stump and did not penetrate to the axons in the proximal nerve stump.

Tissue removal and analysis of the back-labeled neurons

Rats were deeply anesthetized 5-6 days after back-labeling of the neurons and transcardially perfused with 200ml saline followed by 500ml of 4% paraformaldehyde at pH 7.4. After perfusion, the spinal cord was dissected and

spinal cord segments T11 to L2 (containing the CP motoneuron pool) and, L4 and L5 dorsal root ganglia (DRGs) (containing most of the cell bodies of the CP sensory nerves) were harvested and post-fixed with 30% sucrose in 4% paraformaldehyde solution overnight. The tissue was then frozen in liquid nitrogen after been embedded in OCT Tissue-Tek Liquid (Sakura, Japan).

Frozen tissues were sectioned in a cryostat (Jung 3000). Longitudinal spinal cord sections were cut at 50μm thickness and DRG cross-sections were cut at 25μm thickness. The fluorescent bodies of the labeled motor and sensory neurons of the CP nerve were visualized and counted at 40X magnification under fluorescent microscopy at barrier filters of 580 nm (Fig. 4-2). The number of neurons counted was corrected according to the thickness of the sections and the diameter of the neuron cell bodies (30μm and 15μm for motor and DRG neurons respectively) by the method of Abercrombie and Johnson [1]. The correction factor in our samples was 0.635 for motoneuron counts in the spinal cord sections and 0.574 for the DRG sensory neurons.

Morphological analysis of nerve sections

In a third set of rats, surgery was performed to administer rolipram (n=9) or vehicle solution (n=9) immediately after CP nerve transection and repair via an Alzet mini-osmotic pump for periods of 4 or 14 days. The surgeries were the same as described for the previous sets of rats. A segment of the CP nerve 3 mm distal to the suture site was removed at 4 day and 14 day time points. The nerve pieces

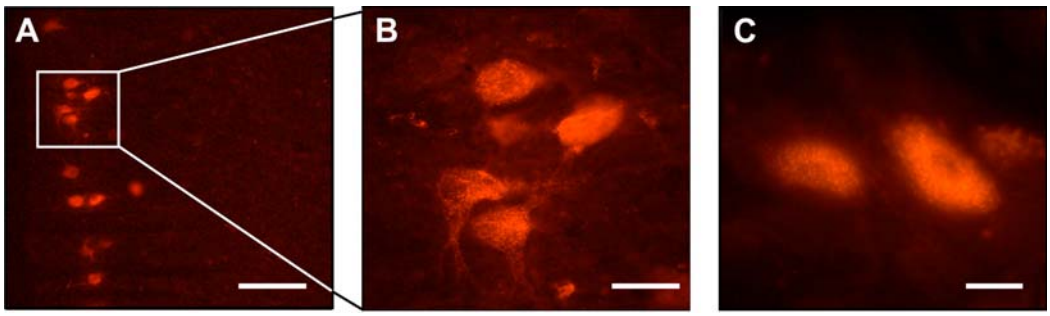

Figure 4-2: Visualization of motor and sensory neurons back-labeled with fluororuby. A,B. An example of a column of common peroneal motoneurons in 50μm thick longitudinal sections of the spinal cord at the lumbar level. The neurons are visualized at low and higher magnification to illustrate the laterally located motoneuron column in the ventral horn and the morphology of the motoneurons, respectively B. Sensory neurons in 25μm thick cross-sections of the L5 dorsal root ganglion. The scale bars are A) 200μm, B) 50μm and C) 20 μm.

were fixed with gluteraldehyde (3% in 0.1M phosphate buffer), stained with osmium tetroxide (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols, and embedded in araldite. Sections of 2μm thickness were stained with methylene blue and observed under the light microscope with a 100X oil-objective lens. In both groups, the number of myelinated fibers with normal appearance and the number of myelinated fibers undergoing Wallerian degeneration were counted to obtain a percentage of degenerative versus intact fibers in the nerve[13]. The number of phagocytic cells present in the cross-section area was also counted.

Measurement of cAMP levels in DRG neurons and spinal cord homogenates

In the rats with the distal nerve stump removed 4 days after nerve transection and repair for morphological analysis (described above), the right L4 and L5 DRGs were removed and immediately frozen in liquid nitrogen. The segment of the spinal cord where L4 and L5 roots enter was also removed, and the left and right sides were separated and frozen in liquid nitrogen. The tissues were homogenized in 0.1N hydrochloric acid (HCl) and cAMP levels were measured with a cAMP immunoassay kit (R&D Systems Inc., MN, USA).

Statistics

Statistical comparisons were performed using SPSS 14.0 software. Comparisons between 2 groups were performed using an independent t-test, whereas comparison between 4 groups were performed using one-way analysis of

variance (ANOVA)**,** and statistical significance was accepted at p values of <0.05 (*) and <0.01 (**). Values are expressed as means ± SE.

Results

Rolipram delivery accelerates motor and sensory nerve regeneration

In order to increase cellular levels of cAMP pharmacologically in motor and sensory neurons, we infused rolipram systemically at a rate of 0.4μmol/kg/h [45] to inhibit PDE IV, the most common isoform of phosphodiesterase in neural tissue[34]. One week after transecting and repairing the nerve, a thin regenerative cord connecting the proximal and the distal stumps was observed through the silicone guide. After 2 to 3 weeks, there was more connective tissue surrounding the guide and the distal stump was obviously thicker.

Elevation of cAMP by rolipram has been shown to increase neurite outgrowth of DRG neurons on a non-permissive substrate *in vitro*[11] but it remains to be determined whether cAMP plays a definitive role in peripheral nerve regeneration *in vivo*. We asked whether rolipram delivery to regenerating axons promotes axon outgrowth across a surgical repair site and into the distal nerve stump. Rolipram was delivered systemically for 7, 14 and 21 days, after which the motor and sensory neurons that had regenerated their axons across the suture site and into the distal nerve stump were back-labeled with fluororuby either 3mm or 10mm distal to the surgical site (Fig. 4-1). When the fluorescent dye was applied at a distance of 3mm from the suture site immediately after nerve repair, the

counts of labeled motoneurons in the spinal cord or sensory neurons in the DRGs were zero. This control experiment demonstrated that the injected retrograde dye was confined within the distal nerve stump. Hence counts of labeled neurons days after nerve transection and repair represented only those neurons whose axons had regenerated across the suture site to the site of dye application. At 7 days after nerve repair, only a few motoneurons were back-labeled with fluororuby 3mm distal to the repair site. In the vehicle saline treated group, a mean (±SE) of 9.1 ± 3.2 motoneurons were back-labeled in the ventral horn of the right spinal cord (Fig. 4-3A) whereas with rolipram treatment, the mean (±SE) number of motoneurons was 32.8 ± 9.3, a significant 3.6 fold increase compared to the vehicle group. There were no back-labeled motoneurons in the spinal cord in 2 of 9 animals in the vehicle group and in 1 of 10 animals in the rolipram group. In other words, there were no motoneurons which regenerated their axons across the surgical site at that time point in ~22% and 10% of the vehicle and rolipram-treated rats respectively. One week later, at 14 days after nerve repair and either vehicle or rolipram treatment, all motoneurons had regenerated their axons to 3mm distal to the suture site in the rolipram treated group with the mean (±SE) number of 343.5 ± 27.1, falling within one SE of the mean number of intact CP motoneurons on the contralateral side of the spinal cord (Fig. 4-3A). In contrast, the mean (±SE) number of motoneurons in the vehicle treated group (210.9 ± 50.6) was significantly lower. Hence, systemic rolipram administration significantly increased the number of motoneurons that regenerated axons across the surgical repair site. When motor axon regeneration through the distal nerve

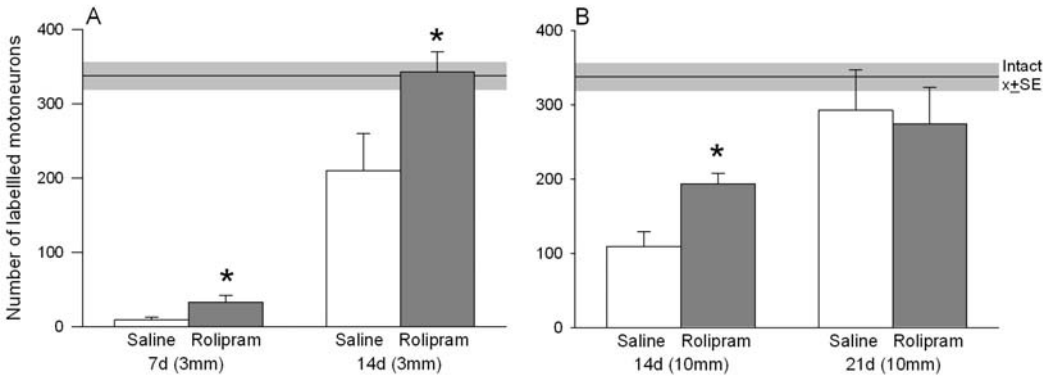

Figure 4-3: Subcutaneous rolipram administration accelerates the onset of motor regeneration after transection and repair of the CP nerve. A. Mean number (±SE) of motoneurons back-labeled 3mm distal to the suture site at 7 and 14 days in the vehicle (white bars) and the rolipram (grey bars) treated groups. B. Mean number (±SE) of motoneurons back-labeled 10mm distal to the suture site at 14 and 21 days in the vehicle and the rolipram treated groups. Rolipram increased the number of motoneurons that regenerated axons across the suture site and into the distal nerve stump at 7 and 14 days with all motoneurons regenerating their axons 10mm from the suture site by 21 days. The numbers that regenerated axons 21 days after saline and rolipram administration were not statistically different from the numbers of contralateral motoneurons that supply axons to the contralateral intact CP nerve. The dashed lines show the SE of the mean number of intact CP motoneurons. *Statistical significance *vs.* vehicle when $p<0.05$.

stump was assessed by back-labeling motoneurons 10mm distal to the suture site at 14 days following transection and repair of the CP nerve, the mean number (±SE) of motoneurons that regenerated their axons through the distal nerve stump was sinificantly higher in the rolipram group (193.6 ± 14.3) as compared to the vehicle treated group (109.1 ± 20.3), a ~2-fold increase (Fig 4-3B). All motoneurons, in both the control and rolipram group, had regenerated their axons by 21d, with mean (±SE) numbers of motoneurons not being significantly different from the number of intact motoneurons in the contralateral intact motoneuron pool (Fig 4-3B). These data are quite consistent with the view that rolipram accelerates axon outgrowth across the suture site and has little or no effect in accelerating the rate of regeneration through the distal nerve stump.

As expected in a nerve that has ~3 times more sensory than motor fibers[39,53], the sensory neuron count in the DRGs were 3X higher than the motoneuron count in the vehicle treated group at each of the different time points after CP nerve transection and repair. There was a positive trend for rolipram treatment to increase the number of DRG neurons that regenerated axons across the suture site at 7 days (66.3±16.8) but the increase was not significant compared to the vehicle treated group (38.3±11.8) (Fig. 4-4A). At 2 weeks and 10mm distal to the suture site, the number of DRG neurons that regenerated their axons in the rolipram group was significantly higher than in the vehicle treated group (403.3±49.4 and 210.9±33.6 respectively) (Fig 4-4B), demonstrating a ~2-fold increase in the number of sensory neurons regenerating axons into the distal nerve

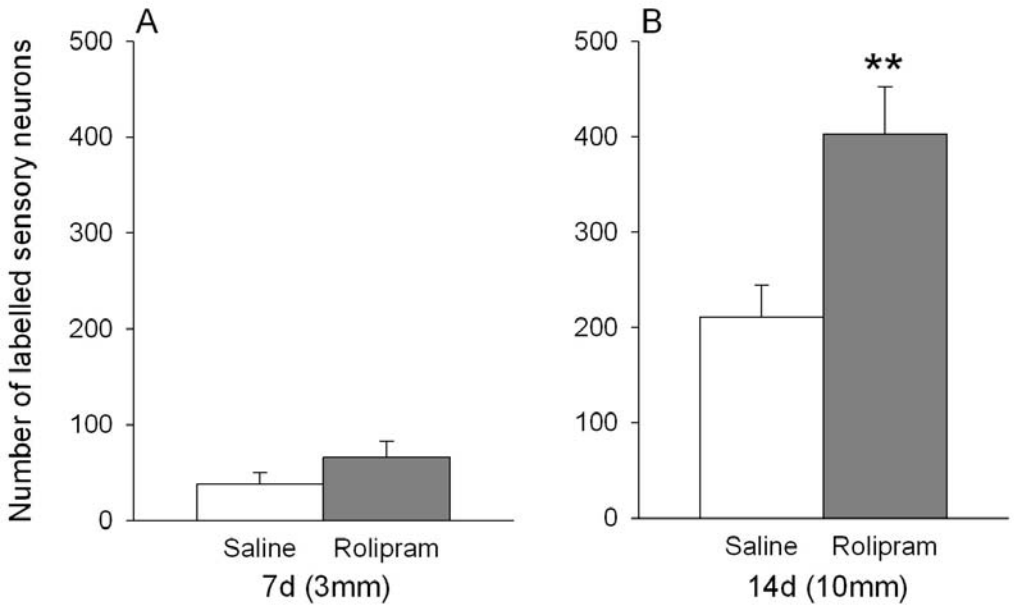

Figure 4-4: Rolipram administration significantly increased the number of sensory neurons regenerating axons across the repair site at 14 days following nerve transection and repair ($p<0.01$). A. Mean number (±SE) of the DRG sensory neurons back-labeled 3mm distal to the suture site at 7 days in the vehicle (white bars) and the rolipram (grey bars) treated groups. B. Mean (±SE) number of DRG sensory neurons back-labeled 10mm distal to the suture site at 14 days in the vehicle and the rolipram treated groups.

stump with rolipram treatment. Thus, rolipram was equally effective in promoting both motor and sensory axon outgrowth across a surgical repair site into the distal nerve stump.

Clear signs of Wallerian degeneration were evident at 4 days as seen in the micrographs of the distal nerve stump (Fig 4-5A,C). At this time point, most of the fibers (67±7%) showed signs of degeneration in the saline treated group (Fig. 4-5A). Some phagocytic cells could be observed in the distal nerve stumps of saline treated rats with mean (±SE) total numbers of 21±7. Rolipram treatment did not alter the proportion of degenerating nerve fibers compared to visually intact fibers (67%±12) nor the number of phagocytic cells (31±9) at 4 days when compared to the vehicle treated group. These findings provide evidence that elevated cAMP levels by rolipram administration are unlikely to affect the rate of Wallerian degeneration. At 14 days after transection and repair of the CP nerve, myelinated axons were observed in 4 of the 5 animals in the vehicle treated group 3mm distal to the suture site, the mean (±SE) number of axons being 98±116. Axons were thinly myelinated and demonstrated the typical morphology of regenerating fibers (Fig. 4-5B). In contrast, all the animals in the rolipram treated group showed myelinated regenerating fibers at 14d (Fig. 4-5D), their numbers being significantly higher at 765±340, an ~8-fold increase. These findings are in agreement with our observations that rolipram treatment increases the number of back-labeled axons that cross the suture site at 7 and 14 days. The relative increase in myelinated fibers seen in the distal nerve stump with rolipram

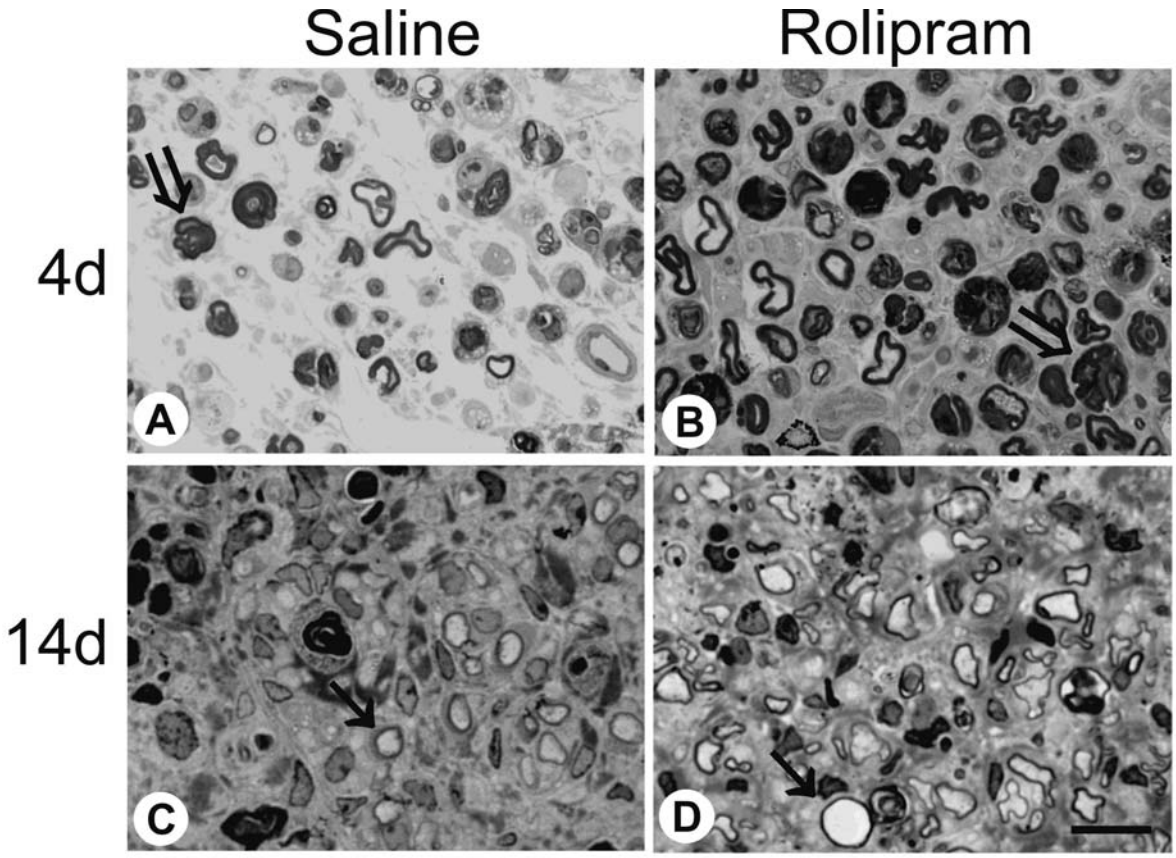

Figure 4-5: Cross-sections of distal nerves 3mm distal to the suture site 4d (A, B) and 14d (C, D) after saline (vehicle) (A, C) and rolipram (B, D) treatment. Quantification of proportions of nerve fibers that had undergone Wallerian degeneration at 4d and of regenerating axons were made, as detailed in the text. Regenerating axons with thinly myelinated sheaths are visible within the distal nerve stumps after CP nerve transection and surgical repair, especially from the rolipram-treated group, at this early time point of axon regeneration. The calibration bar=10μm. Examples of degenerating axons and the regenerating axons are shown by the symbols ⇒ and →, respectively.

treatment at 14 days was 8-fold as compared to the 2-fold increase in the number of sensory and motor neurons extending axons across the suture site and into the distal nerve stump at 14 days (Fig. 3B, 4B). This demonstrates that rolipram promotes the outgrowth of multiple axon sprouts across the surgical repair site in addition to accelerating the number of motoneurons that regenerate their axons across the suture site and into the distal nerve stump.

Chondroitinase ABC promotes peripheral nerve regeneration

ChABC degrades CSPGs present in the endoneurial tissues of the peripheral nerve after nerve injury[36,59]. This degradation occurs without compromising the basal lamina scaffold and does not disturb laminin content. The fact that ChABC injected 2mm distal to the injury site in peripheral nerve accelerates detection of immunoreactive regenerating axons at the site of nerve transection repair, but not after crush injury, strongly indicates that the bacterial enzyme accelerates axon outgrowth rather than rate of regeneration within the nerve stumps [36,59]. We treated the distal CP nerve stump with 20U/ml of ChABC, the same dose that was shown to be effective in promoting motor regeneration in the mouse[16,30] and in degrading CSPGs in the rat sciatic nerve [36,59].

Treatment with ChABC significantly increased the number of motoneurons that regenerated their axons through the distal nerve stump at 14 days post nerve repair as compared with the vehicle saline control (Fig 4-6A). This effect was similar to the effect of rolipram administration over the same

period of time. Hence, ChABC and rolipram, delivered separately, were equally effective in significantly increasing the number of motoneurons that regenerated their axons 10mm into the distal CP nerve stump (Fig. 4-6A). When a combination of one time local ChABC application to the CP nerve and continuous systemic delivery of rolipram over 14 days was performed, the increase in the number of motoneurons regenerating their axons was the same as with the application of each agent alone, there being no additive effect when combining rolipram with ChABC (Fig. 4-6A).

Similar to that observed in motoneurons, ChABC was equally effective in promoting sensory axon regeneration as treatment with rolipram (Fig. 4-6B). Treatment with ChABC significantly increased the number of DRG sensory neurons that regenerated their axons across the surgical repair site (342.3±68.0) when compared to the vehicle treatment group (210.9±33.6) at 10mm distal to the repair site. As observed in the motoneurons, combination therapy of local ChABC and systemic rolipram was not additive in effect. Possible reasons for this are considered in the discussion.

cAMP levels in DRG neurons after systemic rolipram administration

In order to verify that systemic rolipram administration was effective in raising neuronal cAMP levels, we prepared another group of rats where the CP nerve was transected and surgically repaired with either saline solution or rolipram delivered systemically via a mini-osmotic pump over 4 days. After 4

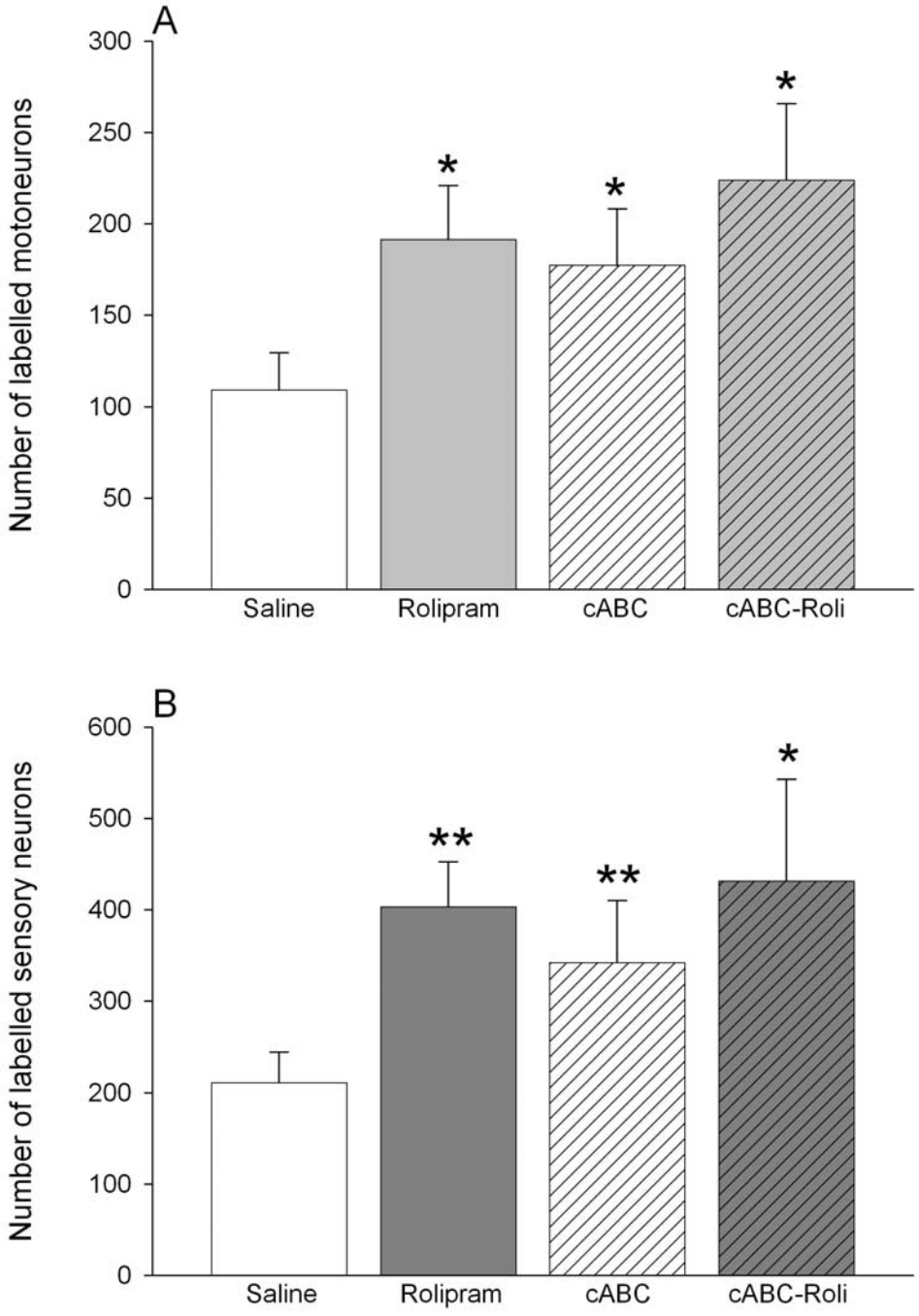

Figure 4-6: Rolipram and ChABC significantly increased the number of motor and sensory neurons that regenerated their axons across the CP nerve transection

and repair site. Combination therapy with both agents did not result in an additive effect above each agent alone. The mean (±SE) number of motoneurons (A) and DRG sensory neurons (B) that were back-labeled 10mm distal to the suture site 14d after the injury in the vehicle treated group (white bar), the rolipram group (grey bar), the ChABC group (white stripped bar) and the group that received both rolipram treatment and ChABC application (ChABC+Roli, grey stripped bar). Rolipram or ChABC significantly increased the number of motoneurons and sensory neurons that regenerated their axons 10mm into the distal nerve stump at 14 days, and the combination of the two treatments had similar effects to each of the two treatments applied separately. *Statistical significance *vs.* vehicle when $p<0.05$ and ** when the $p<0.01$.

days, we harvested the lumbar portion of the spinal cord containing the axotomized CP motoneurons (T12 to L1) and the L4 and L5 DRGs supplying the right hind limb. The corresponding tissues supplying the left hind limb with intact CP nerve were harvested. Rolipram significantly increased cAMP levels in the right hemi-sected portion of the spinal cord containing the axotomized CP motoneurons (212.2±30.7 pmol/ml) when compared to the saline group (87.8± 30.7 pmol/ml) (Fig. 4-7A). The same increase with systemic rolipram treatment was observed in the left hemi-sected cord containing the intact motoneurons compared to the saline control. In the right axotomized DRG sensory neurons, there was a similar significant increase in cAMP but the trend to increase cAMP in the left intact DRG sensory neurons did not reach statistical significance (Fig. 4-7B).

Discussion

Local inhibitors of regeneration in the distal nerve stump of a transected nerve are a temporal impediment to regeneration in the PNS, as Wallerian degeneration rapidly degrades proteoglycans in the extracellular matrix[33] and clears myelin debris[22]. However, the onset of Wallerian degeneration is slowed by the delayed entry of macrophages into the distal nerve stump[8]. We provide evidence here that overcoming the inhibitory effects of myelin by rolipram-mediated elevation of cAMP, or the inhibitory effects of proteoglycans through their degradation by ChABC, accelerates the onset of axon regeneration by promoting motor and sensory axon outgrowth across a surgical repair site.

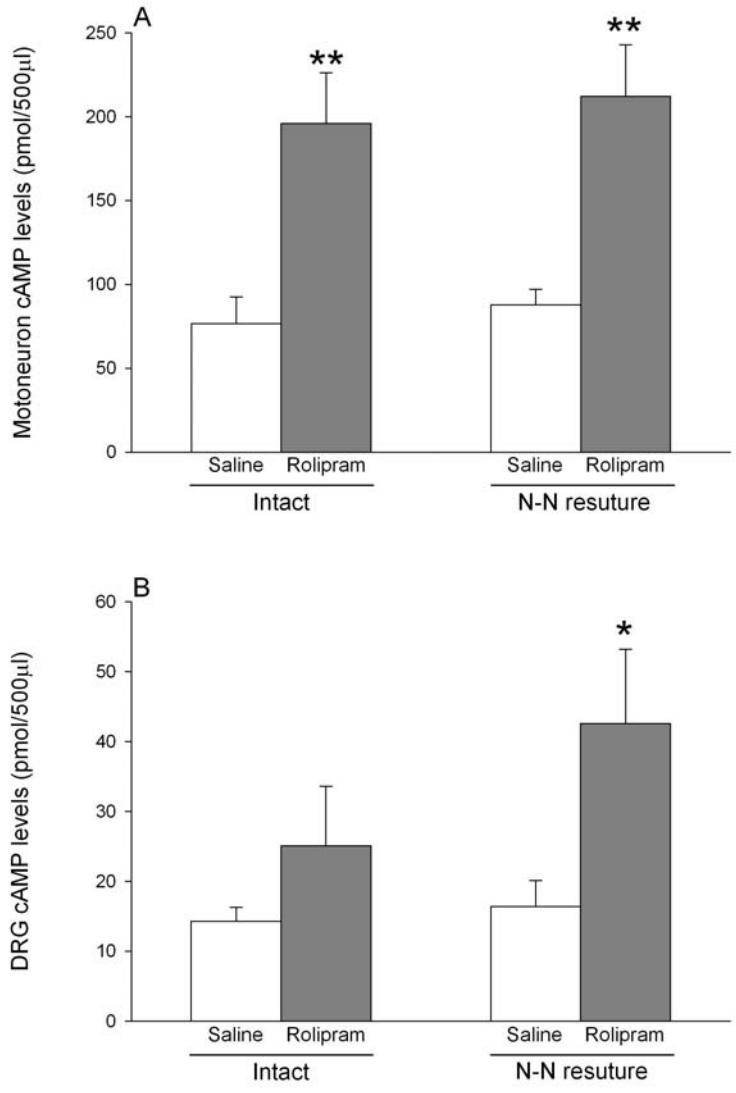

Figure 4-7: Rolipram significantly increased intracellular levels of cAMP in axotomized motor (A) and sensory (B) neurons. Intracellular levels of cAMP from L4-L5 DRG homogenates and from hemi-sected T12-L1 spinal cord segments containing the CP motoneurons from the left intact hind limb were measured. The neurons were axotomized by cutting and resuturing the right CP nerve (N-N resuture) and the neurons on the contralateral side served as intact

controls. Assays were performed 4 days after surgery and treatment with either saline (white bar) or rolipram (grey bar). Rolipram significantly increased cAMP levels in both intact and axotomized motoneurons but only in the axotomized DRG sensory neurons. Mean (±SE) values of cAMP concentrations are plotted in the histograms. Statistical significance $p<0.05$ (*) or $p<0.01$ (**).

Rolipram delivered at the same concentration reported to be effective in CNS axon regeneration[45] accelerated motor and sensory axon outgrowth across a surgical site of peripheral nerve repair. Rolipram administration resulted in a ~3.6-fold increase in the number of motoneurons whose axons had crossed the suture site at 1 week while resulting in a lesser ~2-fold increase in number of motoneurons that regenerated their axons through the distal nerve stump at 2 weeks. The temporal attenuation of effect provides convincing evidence that the primary effect of rolipram is to promote axon outgrowth across a suture site rather than to increase the rate of axon regeneration, similar to that seen with a 1h period of low frequency electrical stimulation of motor and sensory neurons just after surgical repair of a transected peripheral nerve[9,10,25]. Accordingly, studies have reported that increased cAMP levels do not enhance the rate of elongation of peripheral motor[6] and sensory axons[31,40].

The effect of the rolipram in rats essentially mimicked the effectiveness of electrical stimulation in accelerating axon outgrowth in both the PNS and CNS whilst not affecting the rate of axonal extension within the distal nerve stumps [9,10,25,56]. The accelerated axon outgrowth following electrical stimulation has also been linked to increased neuronal cAMP levels[41,56] and thus rolipram and low frequency electrical stimulation may promote axon outgrowth along similar pathways. It is possible that a more sustained increase in cellular cAMP achieved by continuous rolipram administration accounts for a higher percentage of motoneurons that regenerated their axons across the suture site at 2 weeks in the

rolipram treated rats as compared to the application of electrical stimulation for 1h just after nerve repair.

Although effects of systemically delivered rolipram at the site of nerve transection and repair could enhance proliferation of Schwann cells[38,48] and has been reported to accelerate Wallerian degeneration[26], this is unlikely to have made significant contributions to the accelerated outgrowth that was seen at 7 and 14 days after nerve repair and rolipram treatment. Our findings of clear signs of Wallerian degeneration, including the presence of phagocytes and axons undergoing Wallerian degeneration in the distal CP nerve stump, was the same whether or not rolipram was administered.

Rolipram induced elevation of cAMP in axotomized neurons most likely overcomes inhibition from myelin debris and inhibitory CSPGs initially encountered by regenerating axons at the distal nerve stump[50]. In the PNS, probably the main myelin associated inhibitor protein is MAG (myelin associated glycoprotein[50]), whilst NogoR1 (the receptor of Nogo) is not present in regenerating peripheral axons. In fact, recent findings suggest that NogoR1 plays an important role in macrophage recruitment during Wallerian degeneration and would not adversely affect axon regeneration[14]. Thus rolipram induced elevation of cAMP in axotomized, neurons most likely overcomes inhibition from MAG contained in myelin debris initially encountered by regenerating axons at the distal nerve stump[50]. However, even in the absence of inhibitory molecules,

rolipram-induced elevation of cAMP in motoneurons *in vitro* promotes the outgrowth and elongation of neurites[2]. These recent findings support the view that the effectiveness of rolipram in promoting axon outgrowth *in vivo* is mediated at the neuronal cell body level rather than at the local site of axonal injury. Whilst cAMP promotes Schwann cell division, the same extent of Wallerian degeneration in the rolipram and saline control groups supports the view that the primary effect of rolipram is at the neuronal cell body.

Our finding that proteoglycan breakdown by localized application of ChABC was equally effective in accelerating motor and sensory axon regeneration as rolipram administration, provides evidence that rolipram and ChABC mediate their growth promoting effects at different locations. In contrast to cAMP, less is known about the mechanism(s) through which proteoglycans inhibit axon regeneration[49]. CSPGs are abundant and rapidly accumulate after injury in endoneurial tissue[7]. In the PNS, degradation of CSPGs does not seem to play a major role in nerve crush lesions but accelerates the regeneration of axons across a suture site after repair of a transected nerve[36,59] and improves motor functional recovery[29]. In agreement with previous studies[16,30], we report that local application of ChABC increases the number of motor and sensory neurons that regenerate their axons through the distal nerve stump. Zuo *et. al.* [59] attributed the effects of ChABC to the increased ability of regenerating axons to access the basal laminae of the distal nerve stump, which is left intact. Additionally, ChABC degrades CSPGs without displacing laminin in the extracellular matrix

which further promotes axon outgrowth into the distal nerve stump[5]. We did not examine the extent of CSPG degradation with ChABC treatment in our study, however other studies have reported immunohistochemical evidence of efficient degradation of CSPGs with ChABC treatment[59]. Additionally, we performed a single treatment with ChABC at the time of nerve transection and repair, which would have degraded the CPSGs present in the distal nerve stump. However, ChABC has a relatively short half-life and thus our treatment method would have had limited impact on *de novo* CSPG formation in the distal nerve stump. However, the purpose of our experiment was to degrade CSPGs present in the distal nerve stump immediately after nerve transection and repair, thereby limiting the delay in normal degradation which occurs with the progression of Wallerian degeneration.

Although rolipram and ChABC each promote axon outgrowth following nerve injury and repair, the combination of rolipram and ChABC treatments did not result in an additive promotion of axon regeneration. Possible mechanisms to explain this are: (i)Rolipram induced elevation in neuronal cAMP is sufficient to overcome the inhibitory growth environment such that simultaneous enzymatic proteoglycan degradation by ChABC does not impact the outgrowth of regenerating axons; (ii) The individual effects of rolipram and ChABC result in maximum axonal regeneration such that a combination of the two agents would not result in further improvement; and (iii) Both rolipram and ChABC act at different points in the same signaling pathway. The first mechanism seems

unlikely as if it where true, the individual effect on axonal regeneration of ChABC would have been less than that of rolipram, which was not observed in our data. The second mechanism is plausible, whereby individual effects on axonal regeneration were similar between the two treatment groups individually and in combination, suggesting that a maximum level of regeneration had been reached. In this explanation, both agents act through different mechanisms, rolipram at the neuronal cell body and ChABC at the local site of nerve injury[30,59], to produce the same outcome. The third mechanism is also possible, whereby upregulation of cAMP by rolipram and proteoglycan degradation by ChABC both inactivate the inhibitory Rho/ROCK pathway, thereby promoting axon outgrowth via cytoskeletal formation and organization (Fig 4-8). Thus, the combination of rolipram and ChABC would produce the same end effect on the inhibitory pathway as each of the agents individually.

In summary, we have demonstrated that administration of rolipram, a PDEIV specific inhibitor, immediately following peripheral nerve transection and repair was effective in increasing the number of motor and sensory neurons that regenerated axons across the repair site. Our findings are consistent with this effect being mediated through up-regulation of cAMP at the level of the neuronal cell body. Specific breakdown of CSPGs with ChABC was also shown to increase the number of motor and sensory neurons that regenerated axons across the repair site. The findings that simultaneous rolipram and ChABC did not have additive effects demonstrate that either elevation of cAMP or degradation of CSPGs alone

is sufficient to overcome inhibitory environmental factors and accelerate axon outgrowth.

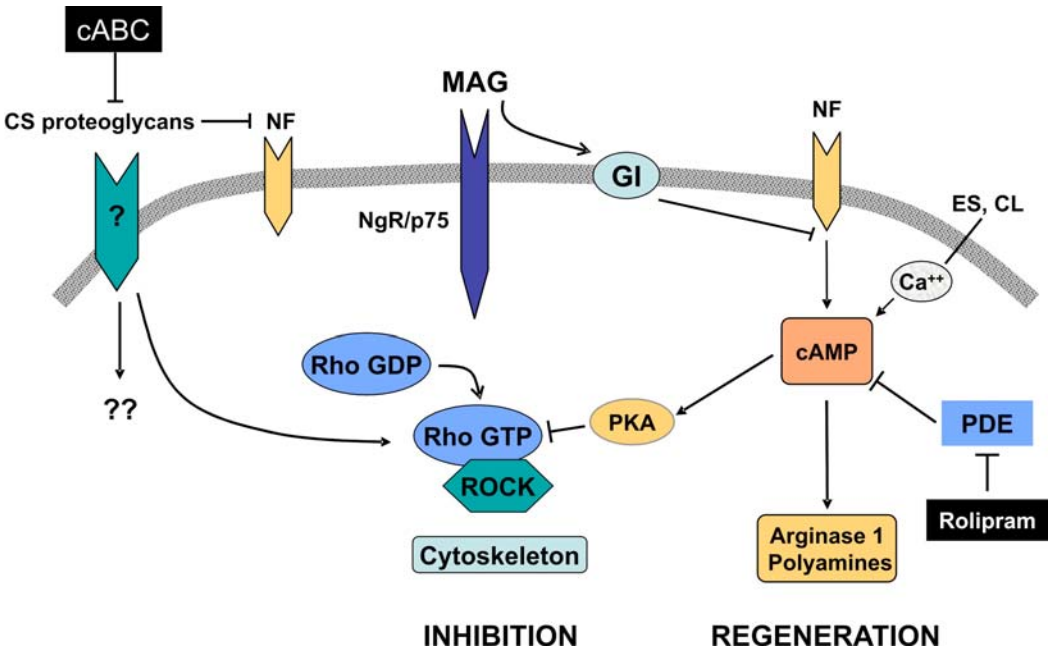

Figure 4-8: Schematic representation of the cross-talk between the inhibitory and regenerative pathways in neurons. Increased cAMP levels by neurotrophic factors (NF), electrical stimulation (ES), a conditioning lesion (CL) or by administration of an inhibitor of phosphodiesterase (PDE) such as rolipram, activate the pro-regenerative pathway and block the pro-inhibitory pathway through phosphokinase A (PKA). Degradation of chondroitin sulfate proteoglycans by local application of chondroitinase ABC (cABC) blocks the inhibitory effects on regeneration induced by proteoglycans[28,42,49,54].

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Chapter 5: Peripheral Nerve Regeneration: Final Discussion

Nerve regeneration in the peripheral nervous system is limited and results in significant patient morbidity. There are two main obstacles to regeneration in the PNS: axonal growth through nerve gaps and a limited time period where Schwann cells and the basal lamina remain supportive of regenerating axons[1,8,12,14]. Through a systematic review of current approaches to nerve repair, different treatment strategies have been reported to overcome these obstacles. Broadly, these strategies fall into pharmacological, surgical and biological approaches. Though broad in scope, this thesis has aimed to explore each of these treatment strategies in a concerted effort to tie commonalities between them. Regardless of treatment, the overall goal in peripheral nerve repair is to facilitate timely axon regeneration to target muscle. Thus the goal in treatment is to either promote quicker axonal outgrowth or maintain a supportive distal environment for longer periods of time in order to facilitate axon regeneration over longer distances or through long nerve gaps. This is achieved mainly through Schwann cell and basal lamina enhancement.

Proximal nerve injuries result in a long regenerative distance whereby the distal stump of the transected nerve is often unable to maintain structure and function conducive to functional reinnervation[8]. To address this, studies have investigated the use of alternative nerve repair techniques including end-to-side nerve repair[15,17,18]. There is little written on side-to-side nerve grafts to bridge a

donor nerve to a denervated recipient nerve and thus we aimed to investigate the feasibility of using side-to-side nerve grafts as a supportive adjunct to improve functional recovery. These lateral nerve connections were able to induce and support axon growth from a donor nerve into a denervated nerve stump to maintain structure and function and thus improve overall functional reinnervation of target muscle. Importantly, this technique achieved this with minimal donor site morbidity. The mechanism behind this protection is simple. Axonal contact is required for the maintenance of the basal lamina and Schwann cells of an endoneurial nerve sheath[7,20]. By providing donor axons through side-to-side nerve grafts, the overall structure and function of the denervated nerve stump was maintained, facilitating axon regeneration from the proximal stump of the transected and repaired nerve.

Long nerve gaps present a similar problem as proximal nerve injuries where growth across an acellular nerve bridge is limited[5,10]. A long nerve gap is essentially analogous to a chronically denervated distal nerve stump with no Schwann cells and degraded basal lamina. Thus, the goal in reconstruction of these defects is to provide a graft through which regenerating axons can grow. An ideal graft is one that provides both Schwann cells and basal lamina and thus autologous nerve grafts are considered the standard of treatment, however, this requires sacrifice of a healthy nerve and results in donor site deficit. Numerous studies have investigated the use of artificial nerve guides and have demonstrated improved regeneration with the incorporation of Schwann cells into these

guides[3,13]. A similar limitation exists with the use of Schwann cells, where a healthy nerve must be sacrificed in order to harvest Schwann cells. Thus, more recently, MSCs have been shown to demonstrate Schwann cell properties[4,22]. In this study, we demonstrated that MSCs can assume Schwann cell phenotype and function, both *in vitro* and *in vivo*, however the differentiation process is only approximately 50% efficient. In terms of tissue engineering, MSC purification and homogeneous differentiation are essential unresolved issues before clinical translation can be considered[11].

The regenerative process in the PNS constitutes two phases. First is the inflammatory and degeneration phase, which is then followed by the regenerative phase[8]. Following nerve transection injury, in order for axonal regeneration to occur, the inhibitory components of the distal nerve stump, including CSPGs and myelin debris, need to be cleared via Wallerian degeneration[16,19,21]. This requires some time as it is dependant on recruitment and influx of macrophages[2,6,9]. The aim of the final study of this thesis was to explore whether simultaneous enhancement of both the degenerative and regenerative phases of nerve regeneration through the use of Chondroitinase ABC and rolipram would significantly improve axonal outgrowth across a coaptation site. The findings of this study indicate that both degradation of CSPGs and promotion of axonal outgrowth result in improved axon regeneration however there is no additive effect with simultaneous treatment. Important findings of this study were that regeneration can be improved with one-time pharmacological therapy at the time

of treatment and there possibly exists a common signaling pathway along which both the degenerative and regenerative processes function[21]. Further study into the signaling pathways activated during nerve injury and regeneration is required to fully apply available pharmacological agents.

There have been many advances in understanding and treatment of peripheral nerve injury and regeneration however there exists many unresolved questions. Surgical, biological and pharmacological approaches have all demonstrated promising potential therapeutic strategies along common physiological pathways. However, standardization and efficiency of delivery are two main obstacles preventing clinical translation of current experimental approaches and future work should be cognizant of these requirements.

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