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DEVELOPMENT OF BIOSYNTHETIC CONDUITS FOR PERIPHERAL NERVE REPAIR

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To my patients, who's ordeal have inspired me

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ABSTRACT

Peripheral nerve injuries are often associated with significant loss of nervous tissue leading to poor restoration of function following repair of injured nerves. Although the injury gap could be bridged by autologous nerve graft, the limited access to donor material and additional morbidity such as loss of sensation and scarring have prompted a search for biosynthetic nerve transplants.

The present thesis investigates the effects of a synthetic matrix BD™ PuraMatrix™ peptide (BD) hydrogel, alginate/fibronectin gel and fibrin glue combined with cultured rat Schwann cells or human bone marrow derived mesenchymal stem cells (MSC) on neuronal regeneration and muscle recovery after peripheral nerve injury in adult rats.

In a sciatic nerve injury model, after 3 weeks postoperatively, the regenerating axons grew significantly longer distances within the conduits filled with BD hydrogel if compared with the alginate/fibronectin gel. The addition of rat Schwann cells to the BD hydrogel drastically increased regeneration distance with axons crossing the injury gap and entering into the distal nerve stump. However, at 16 weeks the number of regenerating spinal motoneurons was decreased to 49% and 31% in the BD hydrogel and alginate/fibronectin groups respectively. The recovery of the gastrocnemius muscle was also inferior in both experimental groups if compared with the nerve graft. The addition of the cultured Schwann cells did not further improve the regeneration of motoneurons and muscle recovery.

The growth-promoting effects of the tubular conduits prepared from fibrin glue were also studied following repair of short and long peripheral nerve gaps. Retrograde neuronal labelling demonstrated that fibrin glue conduit promoted regeneration of 60% of injured sensory neurons and 52% of motoneurons when compared with the autologous nerve graft. The total number of myelinated axons in the distal nerve stump in the fibrin conduit group reached 86% of the nerve graft control and the weight of gastrocnemius and soleus muscles recovered to 82% and 89%, respectively. When a fibrin conduit was used to bridge a 20 mm sciatic nerve gap, the weight of gastrocnemius muscle reached only 43% of the nerve graft control. The morphology of the muscle showed a more atrophic appearance and the mean area and diameter of fast type fibres were significantly worse than those of the corresponding 10 mm gap group. In contrast, both gap sizes treated with nerve graft showed similar fiber size.

The combination of fibrin conduit with human MSC and daily injections of cyclosporine A enhanced the distance of regeneration by four fold and the area occupied by regenerating axons by three fold at 3 weeks after nerve injury and repair. In addition, the treatment also significantly reduced the ED1 macrophage reaction. At 12 weeks after nerve injury the treatment with cyclosporine A alone or cyclosporine A combined with hMSC induced recovery of the muscle weight and the size of fast type fibres to the control levels of the nerve graft group.

In summary, these results show that a BD hydrogel supplemented with rat Schwann cells can support the initial phase of neuronal regeneration across the conduit. The data also demonstrate an advantage of tubular fibrin conduits combined with human MSC to promote axonal regeneration and muscle recovery after peripheral nerve injury.

Key words: Biosynthetic conduit; Mesenchymal stem cells; Nerve graft; Nerve tissue engineering; Peripheral nerve injury; Schwann cells.

ORIGINAL PAPERS

This thesis is based on the following papers which are referred to in the text by Roman numerals.

I. **McGrath AM**, Novikova LN, Novikov LN, Wiberg M. BD™ PuraMatrix™ peptide hydrogel seeded with Schwann cells for peripheral nerve regeneration. *Brain Research Bulletin*, 2010; 83:207-213.

II. Pettersson J, Kalbermatten DF, **McGrath AM**, Novikova LN. Biodegradable fibrin conduit promotes long-term regeneration after peripheral nerve injury in adult rats. *Journal of Plastic, Reconstructive & Aesthetic Surgery*, 2010; 63: 1893-1899.

III. Pettersson J, **McGrath AM**, Kalbermatten DF, Novikova LN, Wiberg M, Kingham PJ, Novikov LN. Muscle recovery after repair of short and long peripheral nerve gaps using fibrin conduits. *Neuroscience Letters*, 2011; 500: 41-46.

IV. **McGrath AM**, Brohlin M, Kingham PJ, Novikov LN, Wiberg M, Novikova LN. Fibrin conduit supplemented with human mesenchymal stem cells and immunosuppressant supports regeneration after peripheral nerve injury. *Neuroscience Letters*, 2012; 516:171-176.

V. **McGrath AM**, Wiberg R, Brohlin M, Kingham PJ, Novikov LN, Wiberg M, Novikova LN. Long term effects of fibrin conduit with human mesenchymal stem cells and immunosuppresion after peripheral nerve repair in a xenogenic model. Manuscript.

ABBREVIATIONS

αMEM	Alpha-modified minimum essential medium
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CsA	Cyclosporine A
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DPX	A mixture of distyrene, plasticizer and xylene
DRG	Dorsal root ganglion
ECM	Extracellular matrix
ED1	Marker specific for activated microglia/macrophages
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
FK506	Tacrolimus
GAP 43	Growth Associated Protein 43
GFAP	Glial fibrillary acidic protein
GDNF	Glial cell derived neurotrophic factor
HBSS	Hank's balanced salt solution
hMSCs	Human mesenchymal stem cells
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
NG	Nerve graft
NGF	Nerve growth factor
NIH	National Institutes of Health
NK	Natural killer cells
NT-3	Neurotrophin-3
OEC	Olfactory ensheathing glial cells
p75	Low-affinity NGF p75 receptor
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PDL	Poly-D-lysine-coated

PHB	Poly-β-hydroxybutyrate
PKH26	Lipophilic membrane dye
PNS	Peripheral nervous system
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
RNA	Ribonucleic acid
S100	S100 protein
SEM	Standard error of the mean
SKPs	Skin derived precursors
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

INTRODUCTION

Clinical background

Epidemiology of peripheral nerve injury

Peripheral nerve injury is a significant cause of functional morbidity, healthcare expenditure and social, avocational and vocational difficulties. Approximately one quater of major nerve injuries occurring in the upper extremity lead to loss of employment (Dagum, 1998; Jaquet et al., 2001). Peripheral nerves injuries can occur in all patients regardless of their age, from perinatal injuries causing obstetric brachial plexus palsy in newborns (Bhat et al., 1995) to elderly patients. The most frequently injured patient is male, younger than 55 years old, with an injury to the upper limb (Taylor et al., 2008). In Sweden, the incidence rate for peripheral nerve injuries is 13.9 per 100,000 person-years and the majority of injuries were found to occur at the wrist and hand level (Asplund et al., 2009). Despite being the most prevalent of etiologies, trauma is not the sole cause of peripheral nerve injuries (Ciaramitaro et al., 2010). Peripheral nerves are vulnerable to a variety of iatrogenic injuries including cancer surgery (Eberl et al., 2011; Navarro-Vicente et al., 2012; Prielipp et al., 2002) and even self harm (Sonmez et al., 2010). The nerve injuries present a spectrum from minor, distal injuries such as digital nerve injuries (Thorsen et al., 2012), via single nerve trunk injuries to brachial plexus injuries resulting in major disability and treatment costs (Lad et al., 2010). When upper limb injuries are considered, the nerve most commonly injured is the radial nerve, followed by ulnar and median nerves (Noble et al., 1998). Lower limb peripheral nerve injuries occur less commonly. The nerve most commonly injured in the lower limb is the sciatic nerve followed by peroneal nerve (Noble et al., 1998).

The outcome of peripheral nerve injuries

The recovery from peripheral nerve injury correlates with numbers of regenerating axons reaching correct target motor and sensory organs and on subsequent maturation and myelination of axons. Although most studies dealing with clinical outcome of peripheral nerve injuries are retrospective, several factors which influence the process of recovery have been identified. These include, but are not limited to, delay between injury and repair, age of the patient, level of the injury and presence of the nerve defect making a primary repair unfeasible (Jivan et al., 2006; Kallio and Vastamaki, 1993; Kallio et al., 1993; Sunderland, 1947; Vastamaki et al., 1993). Delayed nerve repair is performed commonly in clinical practice as partial nerve injuries are frequently explored after a delay of several months if the nerve fails to recover spontaneously (Walsh and Midha, 2009b). Despite significant advances of microsurgical nerve repair with fascicular repair and staining to differentiate between sensory and motor axons to facilitate matching, the outcome of surgical repair of nerve injuries remains suboptimal (Birch, 2012; Lundborg, 1988; Millesi, 2000). As sensory re-learning after nerve injury deteriorates after puberty, regaining all qualities of sensation after nerve injury in adults is impossible (Lundborg and Rosen, 2001). Recovery of pre-injury levels of motor and sensory function is unattainable even in the optimal scenario of distal injury, clean transection and immediate repair (Birch, 2012). If a transected nerve cannot be repaired in a tension free manner (Terzis et al., 1975), the treatment of a nerve gap results in even worse functional outcome (Pabari et al., 2010).

Pathophysiology of peripheral nerve injury

As a response to nerve injury with interrupted axonal continuity, changes occur both proximally and distally to the transection site. The process known as Wallerian degeneration extends beyond the axon to the Schwann cell envelope and myelin sheath and ultimately to the motor and sensory end organs. Wallerian degeneration starts almost immediately after the insult to the axonal continuity and lasts 3 – 6 weeks (Koeppen, 2004). The disintegration of axons starts during the first 24 to 48 hours (Rotshenker, 2011) by appearance of granulation within axoplasma caused by proteolysis of microtubules and neurofilaments secondary to intracellular calcium influx. The disintegration of microtubules leads to interruption of axonal transport. The disintegration of neurofilaments is usually completed within 7 – 10 days. The Wallerian degeneration, although predominantly distal, occurs even proximally towards the node of Ranvier nearest to the injury site. Other cell types essential to the process of Wallerian degeneration are Schwann cells and macrophages, which arrive at the lesion site long before axonal fragmentation (Rosenberg et al., 2012). After loss of axonal contact, the denervated Schwann cells change phenotype from 'supportive' to 'reactive' and proliferate within their basal lamina to form longitudinal bands of Büngner, aligning to receive regenerating axons. Schwann cells secrete cytokines such as interleukin-1β, leukemia inhibitory factor, and monocyte chemoattractant protein-1, which recruit macrophages into the denervated nerve stump (Walsh and Midha, 2009b). Apart from orchestrating the events at the injury side, both Schwann cells and macrophages produce cytokines, neurotrophic factors and ECM molecules to guide and support regenerating axons (Perry and Brown, 1992). Unless axonal contact is re-established, this growth supportive environment cannot be maintained as chronically denervated Schwann cells lose their ability to express regeneration-assisting genes (Gordon et al., 2011; Midha et al., 2005; Sulaiman and Gordon, 2009; Sulaiman et al., 2002a; Walsh and Midha, 2009b; You et al., 1997).

Proximally, there are both structural and functional changes leading to retrograde neuronal degeneration with decreased proximal conduction velocity and change in expression of receptors for neurotrophic factors within hours to days after injury (Birch R, 2012; Fu and Gordon, 1997). The response of the neuronal cell body to axonal disruption ranges from initiation of cell death of sensory neurons and motor neurons (in proximal injuries and an immature nervous system) to several morphological and phenotypic changes in the surviving cell bodies. Dissolution of the rough endoplasmatic reticulum is manifested as chromatolysis (Kreutzberg, 1995). The centrally occurring changes to the dendrites involve their degeneration with stripping of synaptic contacts and functional isolation of the neuron by activated astrocytes and microglia (Brannstrom et al., 1992; Brannstrom and Kellerth, 1998; Novikov et al., 2000). The phenotypic changes from the "signaling mode" to "regenerative mode" (Fu and Gordon, 1997) affect protein expression levels with up-regulation of growth-associated proteins, such as GAP-43 (Skene et al., 1986), genes responsible for cytoskeletal components (Hoffman and Cleveland, 1988), transcription factors c-fos, c-jun, ATF3, NF*k*B, CREB, STAT, neurotrophic factors NGF, BDNF, GDNF, FGF and their receptors, and cytokines TNF*a*, MCP1(Navarro et al., 2007).

The aftermath of axonal injury affects the end organs. In the denervated muscle both structural, electrophysiological and biochemical changes occur as a result of deprivation of trophic and electrical stimuli from the neurons (Midrio, 2006). The principal structural change is the loss of muscle tissue due to atrophy of the individual muscle fibres, especially fast type fibres (Sterne et al., 1997). The proliferation of the connective tissue cells in the denervated muscle leads to fibrosis and replacement of muscle tissue with fat (Dulor et al., 1998).

Thus, the poor outcome of nerve injuries could be attributed to several factors including the retrograde neuronal degeneration, fiber mismatch during nerve repair, presence of inhibitory molecules in the distal nerve stump and Schwann cell denervation (Sulaiman and Gordon, 2009), atrophy of target organs and negative effects of decreased brain plasticity (Bjorkman et al., 2005; Lundborg, 2000; Taylor et al., 2009).

Bridging the injury gap after peripheral nerve injury

The studies included in this thesis focus on gap injuries, where coaptation of proximal and distal stumps directly is impossible because of tissue loss or loss of elasticity of the nerve when repair would have occurred under tension. Autologous nerve grafts are still considered the 'gold standard' for bridging nerve gaps that are not amenable to primary end-to-end repair. Nerve grafting involves sacrificing a less important, sensory nerve to repair the gap in a cable manner. There is an unavoidable morbidity and possible complications associated with donor nerve harvest such as loss of sensation, unsightly scarring, and formation of painful neuroma, cold sensitivity and neuropathic pain. If there is a mismatch between the required amount of nerve grafts and availability of donor nerves, the acceptable alternatives are allografts, acellular nerve grafts, biological nerve conduits composed from vein, muscles or artery or synthetic nerve guidance conduits. Although allografts can promote recovery after peripheral nerve injury, their use may require immunosuppressive treatment, could result in graft rejection and carries a risk of disease transmission. The acellular nerve graft retains the valuable scaffolding of ECM components while at the same time eliminates the risks related to immunogenic response of the host and has been shown to result in functional recovery in gaps up to 20 mm (Mackinnon et al., 2001). Other biological alternatives include veins and arteries, which can be applied in a straight or inside-out manner. Alternatively, acellular muscle grafts contain thick basal lamina, which allows axons to regenerate through the graft. In general, compared with the nerve autograft, axonal regeneration and functional recovery remains unsatisfactory in other biological grafts and does not match autograft performance (Bellamkonda, 2006).

Biosynthetic nerve conduits

A synthetic nerve guidance conduit can be applied to bridge the gap after peripheral nerve injury. One of the first biosynthetic conduits tested experimentally was prepared from a silicone tube (Lundborg et al., 1982a; Lundborg et al., 1982b). Tubular nerve guides can direct axons from the proximal to the distal stump, minimize the infiltration of axonal pathway with scar tissue and their properties such

as length, diameter, rigidity, permeability and biodegradability can be adjusted to suit the injury (Bellamkonda, 2006). Because of fears that the non malleable, non biodegradable silicone conduit produces subsequent nerve compression, other biomaterials were tested resulting in development of several conduits with a few of them approved for clinical use by Food and Drug Administration and Conformit Europe. Several biodegradable conduits, composed from either collagen or polymers derived from synthetic aliphatic polyesters, such as poly(glycolide) or poly(lactide) have been approved for clinical application (Daly et al., 2012; Meek and Coert, 2008; Nectow et al., 2012). In meta-analysis of 33 studies in human application of nerve guidance conduits, there was no statistical difference between tubulization and autologous nerve graft in repairing defects less than 5 cm. Recovery was better for predominantly sensory nerves, with injuries to median nerves with sensory impairment associated with improved postoperative prognosis, while an ulnar nerve with motor nerve damage was prone to a worse prognosis (Yang et al., 2011).
Our group tested wrap-around poly-β-hydroxybutyrate (PHB) implant in patients with median and ulnar nerve injury and found this to be an alternative to epineurial repair (Aberg et al., 2009).

Nerve regeneration through a gap repaired with a hollow tubular conduit can be defined into following stages: (i) the fluid phase; (ii) the matrix phase; (iii) the cellular migration phase; (iv) the axonal phase; and (v) the myelination phase (Belkas et al., 2004). Initially, an influx of plasma exudate filled with neurotrophic factors and extracellular matrix (ECM) precursor molecules from the proximal and distal nerve stumps is followed by the formation of fibrin cable devoid of cells. During the second week after surgery, Schwann cells, endothelial cells and fibroblasts migrate into the fibrin cable (Belkas et al., 2004). Schwann cells proliferate to form aligned glial bands resembling bands of Büngner in the distal stump. Subsequently, axonal sprouts invade the cellular fibrin cable, guided by anisotropic cues. At the conclusion of the axonal phase, Schwann cells switch from proliferative to myelinating phenotype (Deumens et al., 2010).

The ideal tissue-engineered scaffold should offer the most effective strategy to enhance the progression of the neural tissue towards the distal nerve stump ensuring bridging of the nerve defect. Such a hypothetical scaffold should be biocompatible and biodegrade at the controllable rate to allow for varying length of the defect (de Ruiter et al., 2009; Subramanian et al., 2009). The scaffold itself and the by-products of biodegradation should not induce excessive inflammatory reaction. The scaffold should be porous enough (Vleggeert-Lankamp et al., 2007) to allow for early vascularisation and cell migration, while at the same time withstand excessive scarring. The scaffold's 3D structure should mimic the extracellular matrix, enhancing attachment of native Schwann cells or other cell types transplanted in the conduit and provide mechanical guidance cues to the axons extending to bridge the defect (de Ruiter et al., 2009). Additionally, the ability to encapsulate and release growth factors is beneficial (Subramanian et al., 2009). Currently, biomaterial science offers a wide range of biopolymers, synthetic polymers and blends suitable for applications in neural regenerative medicine. Biocompatible biopolymers like keratin, collagen, chitosan, fibrin and alginate can encapsulate growth factors (Nectow et al., 2012). Their degradation profiles and mechanical strength can be

influenced and adjusted to the needs of nerve graft replacement. Synthetic polymers like PCL (polycaprolactone), PHB (poly-β-hydroxybutyrate) or PLGA (poly-lactic-co-glycolic acid) allow the options for extensive tailoring of their degradation, mechanical properties, porosity, and microstructure properties. They allow for encapsulation and release of ECM molecules and growth factors in a timely and spatially controlled manner. Their disadvantage is lower biocompatibility when compared to biopolymers (Nectow et al., 2012). Blends of synthetic materials with biopolymers reduce inflammatory response to conduit implantations and one of the strategies is to combine or ‘‘blend’’ synthetic materials with other more biocompatible materials, such as chitosan or collagen with PLA (Xie et al., 2008).

Another factor influencing successful conduit design is the presence of the extracellular matrix (ECM) molecules which are known to regulate several aspects of neuronal development, including axonal pathfinding, synapse formation and cell survival and migration (Venstrom and Reichardt, 1993). The addition of ECM molecules such as laminin or fibronectin to poly-β-hydroxybutyrate (PHB) was found to increase proliferation of Schwann cell and neurite outgrowth in vitro (Armstrong et al., 2007).

Fibrin is a natural ECM protein and has an inherent role in blood clotting and wound healing. It is formed from fibrinogen by the protease thrombin and then polymerised to form a "mesh" in conjunction with platelets over a wound site. Fibrin matrix is biocompatible and biodegradable. Its mechanical stiffness can be altered by changing the concentrations of its components to suit the application (Chen et al., 2006; Lee et al., 2008). Fibrin glue has been successfully tested to seal severed nerve ends in experimental animals (Palazzi et al., 1995; Zeng et al., 1995) and is widely used as an adjunct to epineural repair in brachial plexus surgery and repair of major nerve trunks (Albala and Lawson, 2006). It has been shown to enable local delivery of growth and neurotrophic factors to enhance angiogenesis (Ehrbar et al., 2008) and promote wound healing (Pandit et al., 2000) and function as an extracellular matrix for transplantation of various cells into injured peripheral nerves, skin and bones (Jubran and Widenfalk, 2003; Yin et al., 2001). Our group demonstrated that tubular conduits prepared from fibrin glue can improve short-term regeneration following peripheral nerve injury (Kalbermatten et al., 2009).

Fibronectin is yet another ECM molecule widely expressed in both CNS and PNS with a role in both neural development and regeneration after nerve injury. In the PNS, fibronectin is expressed along the pathways of neural crest migration (Klar et al., 1992; Yamada, 1989). Its interactions with collagen, heparin, fibrin and cell surface receptors of the integrin family are involved in many processes including cell adhesion, migration, thrombosis and embryonic differentiation (King et al., 2010). Nerve injury upregulates the expression of fibronectin and its receptor, both in the axons of the growth cone and surrounding Schwann cells (Lefcort et al., 1992).

Cell-based therapy

Nerve regeneration through the hollow conduit relies on the presence of the host Schwann cells migrating from the proximal and distal nerve stumps (Daly et al.,

2012). The Schwann cells provide the chemical cues to elongating axons and produce basal lamina necessary for mechanical cues. However, native Schwann cells are able to successfully support regeneration through the conduit only over a critical size defect of 10-15 mm in rodents and 3 cm in humans (Daly et al., 2012; Nectow et al., 2012; Walsh and Midha, 2009b). One of the major experimental strategies to improve the functionality of biologically inert hollow conduits is an enhancement with cell transplantation. Such constructs are aimed to mimic the physiological make up of a nerve graft replete with Schwann cells (de Ruiter et al., 2009; Wiberg and Terenghi, 2003). Transplanted cells may be introduced into the conduit with injection into the lumen, suspension within a hydrogel matrix, seeded along intraluminal guidance structures or released from the wall of the external scaffold (Chang and Hsu, 2004; di Summa et al., 2010; Kalbermatten et al., 2008c; Santiago et al., 2009). These cells provide a supportive environment and exert neuroprotective effects as they produce extracellular matrix molecules, integrins and neurotrophic factors (Boyd and Gordon, 2003). The neurotrophic factors NGF, GDNF, BDNF, NT-3,4/5 and CNTF have been demonstrated to enhance peripheral nerve regeneration by stimulating both axonal growth and neuronal survival (Boyd and Gordon, 2003; Gordon et al., 2003; Jubran and Widenfalk, 2003; Ljungberg et al., 1999; Lundborg et al., 1994; Sulaiman and Gordon, 2002; Terenghi, 1999).

Several authors have found transplantation of Schwann cells to be successful in supporting regeneration after gap injury in the peripheral nerve (di Summa et al., 2011; Hood et al., 2009; Jiang et al., 2010; Madduri and Gander, 2010; McGrath et al., 2010). However, when translational applications are considered, Schwann cells possess several disadvantages. Obtaining a sufficient amount of autologous Schwann cells requires sacrifice of a healthy donor nerve coupled with long *in vitro* expansion time (Walsh and Midha, 2009a). It is well acknowledged that an optimal cell type for transplantation should be easily accessible, rapidly expandable in culture and capable of survival and integration within host tissue (Ren et al., 2012; Walsh and Midha, 2009a). The above requirements directed the attention towards stem cells or progenitor cells as therapy candidates. Embryonic neural stem cells or cell lines were found to support regeneration after repair of nerve injuries (Cui et al., 2008), but their use is associated with potential ethical disadvantages, difficulties in obtaining the embryonic tissue and fears of tumour induction (Cunningham et al., 2012). In contrast, adult stem cells seem to be more promising for future cell therapies. Several cell types have been investigated as potentially suitable for transplantation into the peripheral nerve, including olfactory ensheathing cells (Li et al., 2010; You et al., 2011), neural crest stem cells from the bulge area of hair and whisker follicles (Lin et al., 2011), skin derived precursors (SKPs) (Walsh and Midha, 2009a; Walsh et al., 2010), dental pulp derived stem cells (Kawashima, 2012), adipose stem cells (di Summa et al., 2010; Erba et al., 2010; Santiago et al., 2009) and mesenchymal stem cells (Kalbermatten et al., 2008b; Ladak et al., 2011; Matsuse et al., 2010; McGrath et al., 2012; Pan et al., 2006).

Mesenchymal stem cells (MSC) are readily available from bone biopsies obtained with relatively minor morbidity and can be quickly expanded in culture. MSCs have been shown to differentiate into a Schwann cell-like phenotype expressing S100, GFAP and p75 (Tohill et al., 2004) and are capable of myelinating cultured PC12

cells in vitro (Keilhoff et al., 2006). The transplanted MSCs have been shown to differentiate into other supportive cells types, like endothelial-like cells (Aper et al., 2007; Pankajakshan et al., 2012), smooth muscle cells or pericytes (Bexell et al., 2009), with ability to provide an array of growth factors beneficial directly or indirectly to nerve regeneration (Hobson et al., 1997; Sassoli et al., 2012). MSCs are simililar to Schwann cells in supporting axonal regeneration across the nerve gap (Hu et al., 2007), however, the exact mechanisms behind enhanced regeneration is at present unknown, although in situ differentiation into glial phenotype post transplantation has been noted (Zhang et al., 2004; Zhao et al., 2005).

It has been also hypothesized that human mesenchymal stem cells might posses immunomodulatory properties similarly to rodent and porcine MSCs (Atoui et al., 2008a; Atoui et al., 2008b). MSCs are considered to be MHC class I positive and MHC class II negative, which protects them from NK cell-mediated deletion and allows them to escape recognition by CD4+ T-cells. Moreover, the lack of expression of Fas-ligand and co-stimulatory molecules like B7-1, B7-2 or CD-40 is also advantageous (Pittenger et al., 1999). However, despite evidence that human MSCs remain hypoimmunogenic in a xenogenic setting as demonstrated by mixed lymphocyte reaction (Grinnemo et al., 2004), there are conflicting reports on the reaction to MSCs transplantation *in vivo* (Atoui et al., 2008a; Grinnemo et al., 2004; Grinnemo et al., 2006) with some authors demonstrating survival of human MSCs for 8 weeks and lack of immune rejection in an infracted myocardium model while according to others human MSCs survive only in immunodeficient animals or in animals receiving additional immunosuppressive treatment.

Immunosuppressants and nerve regeneration

As the inflammatory cascade is activated post nerve injury, with major activation of interactions between Schwann cells and macrophages, there is evidence that immunologically active cells and treatment with immunosuppressants may affect the critical aspects of PNS regeneration. Immunophilins (IP) are a family of proteins which function as receptors for immunosuppressive agents cyclosporine A, FK506 (tacrolimus), rapamycin, and their nonimmunosuppressive analogues (Sosa et al., 2005). Cyclosporine A binds to a class of IP receptors called cyclophilins, whereas the receptors for FK506 and rapamycin belong to the family of FK506-binding proteins. The immunosuppressant actions of cyclosporin A and FK506 are initiated by binding to immunophilin with subsequent Ca (2+)-activation of protein phosphatase calcineurin in T-lymphocytes (Liu, 1993; Liu et al., 1992). Immunophilins are implicated in several functions, including regulation of mitochondrial permeability, modulation of ion channels stability (Udina et al., 2003), and participation in axonal transport and synaptic vesicle assembly, transcriptional regulation and play a role in neuroprotection (Avramut and Achim, 2003). However, calcineurin does not appear to be the mechanism behind the neurotrophic effects of these drugs, as non-immunosuppressive cyclosporine A, FK506, and rapamycin analogues do not interact with calcineurin and produce neurotrophic effects to aid functional recovery (Sabatini et al., 1997). FK506 was shown to enhance the rate of axonal regeneration (Gordon et al., 2003; Sulaiman et al., 2002b; Udina et al., 2003), to increase collateral sprouting and the number of regenerating myelinated axons (Sulaiman et al., 2002b; Udina et al., 2003).

Cyclosporine A promotes neuroregeneration and neuronal extension via induction of GAP-43 (Strittmatter et al., 1992) although this effect is less pronounced than for FR506 (Gillon et al., 2003).

Against the above background, the present thesis examines further the growth-promoting effects of biosynthetic conduits, conduit matrix, cell transplantation and immunosuppressive treatment for peripheral nerve injury.

AIMS OF THE STUDY

The overall goal of this thesis was to investigate the effects of biosynthetic conduits prepared from BD™ PuraMatrix™ peptide (BD) hydrogel, alginate/fibronectin hydrogel and fibrin glue in combination with cultured rat Schwann cells or human mesenchymal stem cells on regeneration after peripheral nerve injury in adult rats.

The specific aims of the study were:

- To investigate the effects of a BD hydrogel and alginate/fibronectin gel combined with cultured Schwann cells on neuronal regeneration after peripheral nerve injury and repair in adult rats (Paper I).

- To evaluate the effects of a tubular fibrin conduit on neuronal regeneration and muscle recovery after peripheral nerve injury and repair in adult rats (Paper II).

- To compare the efficacy of a tubular fibrin conduit and peripheral nerve graft on muscle recovery following repair of 10 mm and 20 mm sciatic nerve defects in adult rats (Paper III).

- To assess the growth-promoting effects of a tubular fibrin conduit supplemented with human mesenchymal stem cells on axonal regeneration after sciatic nerve injury and repair in adult rats (Paper IV).

- To study the effects of a tubular fibrin conduit supplemented with human mesenchymal stem cells on long-term regeneration of spinal motoneurons and muscle recovery after peripheral nerve injury and repair in adult rats (Paper V)

MATERIALS AND METHODS

Experimental animals

The experiments were performed on adult (10-12 weeks) and neonatal (1-2 days) female Sprague-Dawley and Fisher 344 rats (Taconic Europe A/S, Denmark; Scanbur AB, Sweden). The animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985) and were also approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were performed under general anesthesia using a mixture of ketamine (Ketalar®, Parke-Davis; 100 mg/kg i.v.) and xylazine (Rompun®, Bayer; 10 mg/kg i.v.).

Cell culture (Papers I, IV & V)

Neonatal Schwann cell culture (Paper I)

Schwann cells were obtained from the sciatic nerves of neonatal rats as described previously (Novikova et al., 2006). The nerves were digested with 1% collagenase I and 0.25% trypsin in Dulbecco's Minimum Eagle's Medium containing Glutamax (DMEM; Invitrogen) for 30 minutes. The digestant was triturated through 21G and 23G needles, filtered through a 70 µm Falcon cell filter (Labora) and centrifuged at 800 rpm for 5 minutes. The cell pellet was re-suspended in DMEM containing Glutamax and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) plus 10% fetal calf serum (FCS, PAA Laboratories). The cells were plated on 25 cm^2 poly-D-lysine-coated culture flasks and kept at 37°C, 95% humidity and 5% CO_2. The next day, incubation for 48 hours in a basic medium containing 10 µM cytosine-b-D-arabinofuranoside (Sigma-Aldrich) was initiated to stop fibroblast growth. Following this procedure, the Schwann cells were in majority in the flasks. The medium was changed to growth medium containing recombinant human glial growth factor 2 (rhGGF2; 40 ng/ml; Acorda Therapeutics) and 10 µM forskolin (Invitrogen). Once the Schwann cells became confluent, the final stage of purification was carried out. The cells were detached with 1.25% trypsin/EDTA and the suspension was centrifuged at 800 rpm for 5 min. The cells were then re-suspended in 1 ml of medium containing mouse anti-Thy-1 antibody (1:1000; Serotec) and incubated at 37°C for 10 minutes. After addition of 250 µl of rabbit anti-mouse complement (Cedarlane® Laboratories), incubation was continued for 30 minutes. The cells were then washed and grown on poly-D-lysine-coated flasks (Sigma-Aldrich). The medium was changed every 48 hours, and the cells were split 1:3 after reaching confluence. The Schwann cells at passage P3 were used for experiments.

Human mesenchymal stem cell culture (Papers IV & V)

Human bone marrow was obtained from the iliac crest of two healthy donors during reconstructive surgery with informed consent. The human procedures were approved by the Local Ethical Committee for Clinical Research in Umeå University. The bone marrow samples were rinsed through each bone cavity with Modified Eagle´s Medium (αMEM) containing 10% (v/v) foetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) using a 21G needle. The resulting cell

suspension was triturated and centrifuged at 1500 g for 5 minutes. After resuspension of the pellet, the cells were filtered through a 70 um Falcon filter and plated in 75cm2 tissue culture flasks and cultured in 37ºC and 5%CO2.

Culture of adult rat dorsal root ganglia neurons (Paper I)
Cultures of dorsal root ganglia (DRG) neurons were prepared from the adult female Fischer rats. The DRG were harvested and treated with 0.125% collagenase Type IV (Sigma-Aldrich) in Neurobasal™-A Medium (Invitrogen) for 90 minutes. DRG were then transferred to 0.25% trypsin for 30 min in 5% CO_2 at 37ºC. The trypsin was inactivated using 30% FCS. The ganglia were then washed in Neurobasal™-A Medium before mechanical dissociation by gentle trituration using a glass pipette. Dissociated neurons were passed through a 70 µm Falcon cell filter (Labora) to remove non-dissociated cells and myelin debris. The cell suspension was centrifuged at 900 rpm for 5 min and then re-suspended in Neurobasal™-A Medium and centrifuged through 15% bovine serum albumin (Sigma-Aldrich) at 1000 rpm for 10 min to remove non-neuronal cells. Neurons were re-suspended in Neurobasal™-A Medium with B-27 supplement (Invitrogen) and 0.5 mM L-glutamine (Sigma-Aldrich), counted and stored on ice.

Dorsal root ganglion neuron-Schwann cell co-culture (Paper I)
Membrane inserts with a 0.4 µm pore size and 6.5 mm inner diameter (Costar) were coated with 2 µg/ml of laminin (Sigma-Aldrich). The DRG neurons were re-suspended in culture medium and plated at a concentration of 5000 cells/insert. After allowing the DRG neurons to attach for 2 hours, the medium was gently removed and the neurons were covered with either 50µl of plain 0.5% BD hydrogel or with BD hydrogel containing neonatal Schwann cells. To initiate gelation of BD hydrogel, 100µl of DMEM was carefully added on the top of the gel and insertions were incubated at 37°C, 95% humidity and 5% CO_2 for 30 min. The hydrogels were washed three times with 200 µl of DMEM during 2 hours, changing only 2/3 of medium every time. After washing, 100µl of Schwann cell growth medium was added into each insert. The inserts were placed in a 24-well plate containing 300µl of DRG culture medium per well and incubated at 37°C, 95% humidity and 5% CO_2 for 36h.

Labelling of cultured Schwann cells with BrdU (Paper I)
To track grafted Schwann cells *in vivo*, the growth medium was supplemented with 10µM 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) at 48 hours before transplantation.

Labelling of cultured mesenchymal stem cells with PKH26 (Paper IV)
Mesenchymal stem cells (passage 2) were fluorescently labelled prior to transplantation in order to enable detection of donor cells in vivo. Approximately 1×10^7 MSC were washed once with αMEM supplemented with 1% (v/v) penicillin–streptomycin (Invitrogen) and centrifuged at 1500 x *g* for 5 min, after which the supernatant was aspirated to leave no more than 25 µl in the pellet. According to the manufacturer's recommendations, the cells were resuspended in 1 ml of diluent C solution (included in the PKH26 Red Fluorescent Cell Linker Kit, Sigma-Aldrich) and then incubated in 4 µM PKH26/1 ml dilutent C dye solution for 5 min. The

labelling reaction was stopped by adding 2 ml of FBS. Labelled cells were washed two times with αMEM/FBS by centrifugation for 10 min at 1500 x *g*. Labelled cells were then evaluated under a fluorescent microscope.

Preparation of biosynthetic conduits

Millipore membrane conduit (Paper I)

A cellulose membrane with filtration cut off point of 10 000 Daltons (Millipore) was cut into rectangles measuring 14x8 mm, molded around a cannula with an external diameter of 2.2 mm and welded at 250ºC. Membrane conduits were soaked in 70% alcohol for 24 hours and then carefully rinsed in several portions of normal saline. The conduits were filled with plain hydrogels or hydrogels containing Schwann cells immediately before the surgery.

BD Pura Matrix peptide hydrogel with Schwann cells (Paper I)

To prepare a cell carrier from BD™ PuraMatrix™ peptide hydrogel (BD hydrogel; BD Biosciences), 1% stock solution was mixed with 20% sucrose (Sigma-Aldrich) and DMEM containing Ca^{2+} and Mg^{2+} in proportion 2:1:1, which resulted in 0.5% hydrogel. To prepare hydrogel containing cultured cells, Schwann cells were detached from the culture flasks with trypsin/EDTA, washed and assessed for viability with trypan blue (Sigma-Aldrich). The cells were concentrated in their own growth medium, re-suspended in 20% sucrose to prevent gel formation and added to the hydrogel so that after mixing with the hydrogel a final concentration of $80x10^6$ cells/ml was obtained (Mosahebi et al., 2001). The solution was left for 1 minute, mixed gently with a pipette and the resulting hydrogel was used for experiments. After loading the matrix into the lumen, the conduits with BD hydrogels were washed *in situ* with HBSS containing Ca^{2+} and Mg^{2+}.

Alginate/fibronectin matrix with Schwann cells (Paper I)

To prepare a cell carrier based on ionically cross-linked alginate hydrogel, a sterile stock solution of 4% w/v ultra pure low-viscosity mannuronic acid alginate (Pronova) in 150 mM of NaCl was mixed with 0.1% liquid fibronectin (Sigma-Aldrich) in proportion 1:1, which resulted in 2% alginate containing 0.05% fibronectin. To prepare hydrogel containing cultured cells, Schwann cells were detached from the culture flasks and concentrated in their own growth medium. The cells were re-suspended in 0.1% fibronectin and added to the hydrogel to obtain a final concentration of $80x10^6$ cells/ml (Mosahebi et al., 2001). After loading the matrix into the lumen, the conduits with alginate/fibronectin hydrogels were treated with 100 µl of 0.1 M $CaCl_2$ solution for 2 min and then washed with DMEM.

Fibrin glue conduit (Papers II-V)

Tubular fibrin conduit was molded from two compound fibrin glue (Tisseel® Duo Quick, Baxter). Fibrin glue contains; 70-110mg/ml fibrinogen, 2-9mg/ml of plasma fibronection, 10-50 U/ml of factor XIII, 40-120 ug/ml of plasminogen, 3000 KIU/ml of aprotinin solution, 5 IU/ml of thrombin and 40mmol/l of calcium chloride. All components were mixed in sterile conditions according to manufacturer recommendations. A silicone mold with a centrally placed metal rod was used to prepare tubular 14-mm-long conduits with uniform 1-mm-thick walls and 2mm lumen. A similar silicone mold with different dimensions was used to create 24-mm-

long conduits with a 1mm-thick wall at the proximal end, which became 2mm-thick at the distal end, with a uniform 2mm lumen (Paper III). After glue polymerization, the rods and silicone mold were removed and the fibrin glue conduits were stored in sterile Dulbecco's Minimum Eagle's Medium at room temperature.

Fibrin matrix with human mesenchymal stem cell (Papers IV & V)

Fibrin matrix was produced by modifying a two compound fibrin glue (Tisseel® Duo Quick, Baxter) which were diluted according to Bensaid et al (Bensaid et al., 2003a). To dilute the fibrinogen 1:5 a buffer consisting of 10 ml of de-ionised water with 73.5 mg of sodium citrate, 16.9 mg sodium chloride, 249.9 mg glycine, 30 000 KIU aprotinin (Sigma, A-3428, Sigma-Aldrich) and 150 mg albumin serum (Sigma, A-3428, Sigma-Aldrich). For dilution of thrombin, a solution of 10 ml of de-ionised water with 58.8 mg calcium chloride, 87.1 mg sodium chloride, 30.3 mg glycine and 500 mg serum albumin was prepared and diluted 1:5. The two solutions were then combined as explained below producing a relatively soft fibrin clot (Bensaid et al., 2003b) with 18 mg of fibrinogen per ml and 100 IU of thrombin per ml generating the optimal solution of 1/100 dilution of thrombin and 1/10 dilution of fibrinogen.

Sciatic nerve injury model and experimental groups

A three cm skin incision was made over the left gluteal area. Under an operating microscope (Zeiss, Carl Zeiss, Germany), the sciatic nerve was exposed through splitting of gluteal and biceps muscle and then divided 5mm below the exit point from sciatic notch and 7 mm or 14 mm of sciatic nerve distal to the division was removed creating 10 mm or 20 mm gap. In the nerve graft groups a 10 mm (Papers I, II, III & V) or 20 mm (Paper III) long sciatic nerve segment was reversed and a epineural nerve repair was performed using four interrupted 10-0 nylon sutures (S&T Marketing). In the conduit group, the 14 mm or 24 mm long conduit was inserted into the gap, allowing for intubation of the nerve end 2 mm into the conduit, resulting again in 10 mm and 20 mm gap respectively between proximal and distal sciatic stump. The conduit was fixed to the epineurium with one 10.0 Ethilon suture at each end. Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures.

The animals were divided into the following experimental groups: (i) membrane conduit with BD hydrogel alone, (ii) membrane conduit with alginate/fibronectin hydrogel, (iii) membrane conduit with BD hydrogel and Schwann cells, (iv) membrane conduit with alginate/fibronectin hydrogel and Schwann cells, (v) tubular fibrin conduit alone, (vi) tubular fibrin conduit with fibrin matrix and (vii) fibrin conduit with fibrin matrix and human MSCs. The operated animals were allowed to survive for 3 weeks (Papers I & IV), 12 weeks (Paper V) or 16 weeks (Papers I, II & III).

Treatment with cyclosporine A (Paper IV & V)

The animals in experimental groups (vi) and (vii) received daily injections of cyclosporine A (Sandimmum, Novartis) which was injected intraperitoneally at 1.5 mg per 100 g body weight starting from 24 hours before surgery and continued for 3 weeks (Paper IV) or 12 weeks (Paper V), respectively. At week 2 the weight of the animals was measured to ensure consistent delivery of the same concentration of

cyclosporine A. In the long term group the weight measurements were performed on a monthly basis.

Retrograde neuronal labelling (Papers I, II and V):
To study the morphology of axotomized spinal motoneurons in Paper V, the medial gastrocnemius motoneuron pool was pre-labeled with 2% fluorescent tracer Fast Blue (FB, EMS-Chemie GmbH) one week before sciatic nerve injury and repair. In order to identify motor and sensory neurons regenerating through the nerve graft and conduits, the tibial nerve (Papers I, II) or sciatic nerve below the distal nerve-graft interface (Paper V) were labelled with 2% fluorescent tracer Fast Blue (Papers I, II) or 10% fluorescent tracer Fluoro Ruby (Invitrogen; Paper V). The cut tibial nerve was placed into the tube with Fast Blue solution and the open end of the tube was sealed with a mixture of silicone grease and Vaseline to prevent leakage. The distal end of the tube was fixed to the surrounding tissues with Histoacryl® glue (B.Braun Surgical GmbH). The cut sciatic nerve was labelled by applying a small cube of Spongostan (Ethicon) drenched in 10% aqueous solution of fluorescent tracer Fluoro Ruby to the proximal stump. The nerve was isolated from the surrounding tissue by a well from mixture of silicone grease and Vaseline. Two hours later the tracer was removed, the nerve was rinsed in normal saline and the wound closed in layers. The animals were left to survive for one more week to enable labelling of the neurons before the termination of the experiment.

Tissue processing
Schwann cells cultured in BD hydrogel on membrane inserts and mesenchymal stem cells cultured on Lab-Tek® slides were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at room temperature for 15-20 minutes. The animals were deeply anaesthetized with an intraperitoneal overdose of sodium pentobarbital and transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The spinal cord segments L4–L6 with corresponding DRGs were removed and post fixed overnight in the same fixative. For neuron counts, spinal cord segments were cut in serial 50-µm-thick parasagittal sections on a Vibratome (Leica Instruments), mounted onto gelatin-coated slides and coverslipped with DPX. The DRGs were cryoprotected in 20–30% sucrose for 2–3 days at 4ºC, embedded in Tissue-Tek (O.C.T., Miles Inc), frozen at -80°C, cut in serial 40µm thick sections on a cryomicrotome (Leica Instruments), mounted on gelatin-coated slides and coverslipped with DPX. The conduits were postfixed for 2h, cryoprotected in 10% and 20% sucrose for 48 hours and frozen in liquid isopentane. Serial longitudinal 16-µm-thick sections were cut on a cryomicrotome, thaw-mounted in pairs onto SuperFrost® Plus slides, dried overnight at room temperature and stored at -85ºC before processing. For morphometric analyses and axon counts, 2-3 mm long sciatic nerve specimens were excised at 3-5 mm distance from the distal nerve-graft interface. The nerves were additionally fixed in 3% glutaraldehyde, postfixed in 1% OsO4 in 0.1 M cacodylate buffer (pH=7.4), dehydrated in acetone, and embedded in Vestopal. Semithin transverse sections were cut on a 2128 Ultratome (LKB, Sweden) and counterstained with Toluidine Blue. Using the operating microscope, the entire gastrocnemius and soleus muscles were cleaned and dissected out, dividing its tendinous origin and insertion flush with the bone. The muscles were weighed immediately following harvest and then embedded

in OCT compound and snap frozen in liquid nitrogen. Sixteen micron transverse sections of gastrocnemius muscles from the contralateral and operated sides were cut on a cryomicrotome (Leica Instruments), thaw-mounted in pairs onto SuperFrost®Plus slides, dried overnight at room temperature and stored at -85°C before processing. Sections were fixed with 4% paraformaldehyde for 15 min before immunohistochemical staining.

Immunohistochemistry

After blocking with normal serum, the following primary antibodies were used: rabbit anti-5-bromo-2-deoxyuridine (1:2000; BrdU; Sigma-Aldrich), rabbit anti-S-100 protein (1:1000; Dakopatts), mouse anti-βIII tubulin (1:1000, Sigma-Aldrich), mouse monoclonal anti-ED1 (1:100, Abcam), monoclonal primary antibodies raised against fast and slow myosin heavy chain protein (NCL-MHCf and NCL-MHCs, Novocastra, both 1:20 dilution). Human MSC were stained with a human Mesenchymal Stem Cells Characterization Kit (Millipore) for CD14, CD19, H-CAM (CD44), Thy1.1 (CD90) and MCAM (CD146) according to the manufacturer's recommendations and mouse anti-CD54 (1:20, Millipore), rabbit anti-CD105 (1:20, R&D Systems), mouse anti-collagen type IV (1:200, Millipore) and mouse anti-fibronectin (1:500, Millipore). All primary antibodies were applied for 2 hours at room temperature. After rinsing in PBS, secondary goat anti-mouse, goat anti-rabbit antibodies Alexa Fluor® 488 and Alexa Fluor® 568 (1:300; Molecular Probes) or Northern Lights 557-conjugated Streptavidin (1:10 000, R&D Systems) were applied for 1-2 hours at room temperature in the dark. The slides were coverslipped with Vectashield® mounting medium containing DAPI (Vector Laboratories). The staining specificity was tested by omission of primary antibodies.

RT-PCR for neurotrophic factors (Paper V)

The RNeasy™ mini kit (Qiagen) was used according to the manufacturer's protocol for the isolation of total RNA from the cell pellets of the human MSC and then 1ng RNA was incorporated into the One-Step RT-PCR kit (Qiagen) per reaction mix. Primers were manufactured by Sigma, UK. A thermocycler (Biometra, Germany) was used with the following parameters: a reverse transcription step (50ºC, 30min), a nucleic acid denaturation/reverse transcriptase inactivation step (95ºC, 15min) followed by 35 cycles of denaturation (95ºC, 30sec) and annealing (30sec, optimised per primer set) (see Paper V, Table 1) and primer extension (72ºC, 1min) followed by final extension incubation (72ºC, 5min). PCR amplicons were electrophoresed (50V, 90min) through a 1.5% (w/v) agarose gel and the size of the PCR products estimated using Hyperladder IV (Bioline). Samples were visualised under UV illumination following GelRed™ nucleic acid stain (BioNuclear) incorporation into the agarose.

Morphological analysis

Distance of regeneration in the conduit (Paper I & IV)

Distance of regeneration was measured with a calibrated graticule of a Leitz microscope to the nearest 0.1 mm on longitudinal sections of the conduits from the entry point of the conduit to the furthermost axon stained against βIII tubulin and expressed in micrometers. Distance of regeneration was measured on 3 consecutive sections from each conduit spaced at 50 micrometers apart.

Counts of myelinated axons in the distal stump (Paper II)
Myelinated axons in the distal tibial nerve stamp were counted at x1000 final magnification using a fractionator probe in Stereo Investigator™ 6 software (MicroBrightField, Inc.,USA).

Counts of retrogradely labelled neurons (Papers I, II & V)
Nuclear profiles of labelled spinal motoneurons were counted in all sections at x250 magnification in a Leitz Aristoplan microscope using filter block A. The total number of nuclear profiles was not corrected for split nuclei, since there was uniformity in nuclear size and the nuclear diameters were small in comparison with the section thickness. Furthermore, in estimations of retrograde cell death the accuracy of this technique is similar to that obtained by using physical dissector (Ma et al., 2001) or by counting neurons reconstructed from serial sections (Novikova et al., 1997).

ED1-positive area of staining (Paper IV)
The pictures were taken from the central part of the conduit and 5mm distally and proximally from the middle at x40 magnification. One or more photographs were taken depending on the width of the nerve in the particular specimen. Images were processed with help of Adobe Photoshop CS3, pasted onto a single canvas and trimmed to reconstruct a single image of a band of a standard width perpendicular to the long axis of the conduit or the nerve graft. The area of staining with βIII tubulin and ED1 was calculated after image calibration using Image Pro Plus software (Media Cybernetics) and expressed in square micrometers.

Morphometric analysis of muscle (Paper III & V)
Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz microscope. Morphometric analysis of muscle sections was performed on coded slides without knowledge of their source. Five random fields were chosen (using the x16 objective) and images for the immunolocalisation of each myosin heavy chain type plus that for laminin were captured using the appropriate emission filters, and combined to provide dual-labelled images. Each image contained at least 25 individual muscle fibres for analysis. Image Pro-Plus software was calibrated to calculate the mean area and diameter (in μm) for each muscle. The injured side was expressed relative to the contra-lateral control side and the relative mean % ± SEM calculated for each group.

Image processing
Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz Aristoplan microscope. The captured images were resized, grouped into a single canvas and labelled using Adobe Photoshop CS3 software. The contrast and brightness were adjusted to provide optimal clarity.

Statistical analysis
One-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test or Bonferonni test (Prism®, Graph-Pad Software) was used to determine statistical differences between experimental groups.

RESULTS

Culture of Schwann cells and DRG neurons in BD hydrogel (Paper I)
The compatibility of Schwann cells with BD hydrogel was examined in 3D cultures during 2 weeks. The cells were evenly distributed through the hydrogel and retained their typical spindle-shaped morphology (Fig. 1A). Addition of neonatal Schwann cells to BD hydrogel resulted in increased outgrowth of βIII-tubulin-positive neurites from DRG neurons, when compared with the neurite outgrowth in plain BD hydrogel (Fig. 1B,C).

Effects of BD hydrogel and Schwann cells on axonal growth (Paper I)
Analysis of the conduits revealed the apparent difference in orientation of regenerating βIII-tubulin+ axons and S100 protein+ Schwann cells between BD hydrogel and alginate/fibronectin hydrogel. In the conduits filled with BD hydrogel, both axons and Schwann cells were aligned longitudinally and regeneration within the conduit grossly resembled the morphology of regeneration within the peripheral nerve graft. In contrast, the alginate/fibronectin was concentrated in clumps in certain areas providing obstacles for regenerating axons and migrating Schwann cells. As a result, the arrangement of regenerating axons followed by Schwann cells was irregular. At 3 weeks in the group repaired with a conduit containing plain BD hydrogel, the axons reached a mean distance of 8.56 ± 0.93 mm (mean ± SEM, Table 1). However, the addition of neonatal Schwann cells to BD hydrogel stimulated axonal regeneration to reach 15.88 ± 0.21 mm (mean ± SEM) which corresponds to a 73% increase in the regeneration distance with the axons crossing the 10 mm gap and entering the distal nerve stump for almost 6 mm ($P < 0.001$; Fig. 2F, Table 1). In contrast, the regeneration distance in experimental nerve injury groups repaired with alginate/fibronectin hydrogel alone and alginate/fibronectin hydrogel with cultured neonatal Schwann cells was 4.94 ± 0.22 mm (mean ± SEM) and 4.64 ± 0.11 mm (mean ± SEM), respectively ($P < 0.001$; Table 1). Three weeks after transplantation, numerous BrdU-labelled Schwann cells were present both within the BD hydrogel and alginate/fibronectin hydrogel (Fig. 2D and E). However, in the alginate/fibronectin hydrogels, the Schwann cells had a tendency to form cell clusters whereas in the BD hydrogel they were more evenly distributed throughout the matrix (Fig. 2D and E).

Effects of BD hydrogel and Schwann cells on neuronal regeneration (Paper I)
Retrograde neuronal labelling with fluorescent tracer Fast Blue revealed that in control experiments, at 16 weeks after nerve injury and peripheral nerve grafting 1001 ± 37 (mean ± SEM) tibial motoneurons regenerated their axons 10 mm into the distal stump of the sciatic nerve (Table 2). After transplantation of the membrane conduits containing BD hydrogel and alginate/fibronectin hydrogel, the number of regenerating spinal motoneurons was decreased to 49% and 31%, respectively, when compared with the control group ($P < 0.001$; Table 2). Addition of the cultured Schwann cells to the BD hydrogel and alginate/fibronectin hydrogel resulted in regeneration of 57% and 55% of spinal motoneurons, respectively ($P > 0.05$; Table 2). Therefore, Schwann cells did not significantly improve the number of regenerating neurons.

Effects of BD hydrogel and Schwann cells on muscle recovery (Paper I)
The recovery of gastrocnemius muscle after sciatic nerve injury and repair was expressed as a ratio between ipsilateral and contralateral muscle weights (Table 3). Muscle weight recovered to 59% of the contralateral side after peripheral nerve grafting and it was significantly higher in comparison with all the experimental groups with membrane conduits and hydrogels ($P < 0.001$; Table 3). Although there were no statistical differences between membrane conduits with hydrogels and hydrogels containing Schwann cells, the BD hydrogel was superior in promoting muscle recovery than alginate fibronectin hydrogel ($P < 0.001$; Table 3).

Effects of fibrin conduit on neuronal regeneration (Paper II)
At 16 weeks after implantation, the fibrin conduits were completely reabsorbed. There were no surgical complications in form of inflammation at the repair site. The neo nerve connecting the proximal and distal stump contained a network of small blood vessels. Numerous axons connecting the proximal and distal nerve stumps associated with Schwann cells were arranged in a structure resembling a peripheral nerve as shown on longitudinal sections immunostained with antibodies against βIII-tubulin (axons; Fig 1A, D) and S100 protein (Schwann cells; Fig 1B, E). The axons entered and continued into the distal nerve stump. In the nerve graft repair group 5184 ± 574 (mean ± SEM) retrogradely labelled sensory dorsal root ganglion (DRG) neurons and 1001 ± 37 (mean ± SEM) motor neurons from the tibial pool regenerated into the distal stump (Fig. 2A, C). After implantation of fibrin conduit significantly lower number of regenerating neurons was shown when compared to the nerve graft control group. Repair of the nerve gap with the fibrin conduit resulted in regeneration of 3096 ± 187 (mean ± SEM) sensory DRG neurons ($p< 0.05$; 60% of control, Table 1; Fig. 2B) and 520 ± 85 (mean ± SEM) spinal motor neurons ($p<0.05$; 52% of control, Table 1; Fig 2D). Compared with the number of neurons in the tibial motoneuron pool in unoperated rats (Novikova et al., 1996) implantation of tubular fibrin conduit supported regeneration of approximately 40% of all motoneurons projecting into the tibial nerve.

Effects of fibrin conduit on axonal sprouting (Paper II)
To further investigate the regeneration through the tubular fibrin conduit the number of myelinated axons was examined in the distal stump 3-5 mm from the distal anastomosis. Despite similar appearance and distribution of myelinated fibres in both experimental groups (Fig. 2E and Fig. 2F), analysis of the number of myelinated axons revealed that regeneration through the fibrin conduit resulted in lower axonal counts when compared with the nerve graft group. After reversed nerve graft repair, the distal nerve contained 25,370 ± 686 (mean ± SEM) myelinated axons, while after fibrin conduit implantation the number of axons was decreased to 21,750 ± 1252 (mean ± SEM; 86% of control; $p<0.05$; Table 1). The comparison of the numbers of myelinated axons with regenerating motoneurons revealed that axons in the distal nerve underwent highly significant axonal sprouting after both fibrin conduit and nerve graft transplantation. However, proportionally the implantation of fibrin glue conduit induced higher sprouting in the distal stump than repair of the nerve gap with a reversed nerve graft.

Effects of fibrin conduit on muscle recovery (Paper II & III)
To assess recovery of gastrocnemius and soleus muscles following nerve repair, the ratios between weights of ipsilateral muscles of each of the experimental groups were compared with the contralateral muscle weights. Muscle weight of gastrocnemius and soleus muscles recovered to 59% of the contralateral side following 10 mm gap repair with peripheral nerve grafting (Table 2, Paper II). When these results were compared with muscle weight following fibrin conduit implantation, a similar recovery was found for soleus muscle (89% of nerve-graft control; $p > 0.05$; Table 2, Paper II). Repair of both 10 mm and 20 mm gaps with reversed nerve grafts resulted in similar gastrocnemius muscle weight recovery (Table 2, paper II and Table 1, Paper III).

Analysis of general morphology of the gastrocnemius muscles after nerve graft repair through both gap lengths showed an ordered structure with the muscle fibres grouped in fascicles (Fig 1A, Paper III). Fast type fiber mean area and the mean diameter were measured and no significant differences were found between both 10 and 20 mm gap groups (Fig 1B, Paper III). Similar results were found for the slow type fiber mean area and the mean diameter (Fig 2A and 2B, Paper III). Comparison of fresh gastrocnemius muscle weights in groups where the nerve gap was repaired with fibrin conduit revealed that the muscle weight recovered to 81% of the nerve graft group in the 10 mm nerve gap but only recovered to 43% when compared with the nerve graft group in the 20 mm gap (Table 1, paper III). For the 10 mm gap, similar general muscle morphology was found in both nerve graft repair group and in animals treated with fibrin conduit (Figs 1A and 2A, Paper III). Similarly, in the fibrin conduit group the fast type and slow type muscle fiber area and diameter were comparable to nerve graft in the 10 mm gap injury (Fig 1B and 2B, Paper III). In experimental animals treated with fibrin conduit across the 20 mm nerve gap, clearly smaller muscle fibres compared with the 10 mm nerve gap repairs were found and a more disorganized structure was present (Figs 1A and 2A, Paper III). The analysis of muscle fiber size confirmed the qualitative findings, with fast type fiber mean area and diameter of muscles from the fibrin group, which were significantly ($P<0.01$) lower than the nerve graft group over the 20 mm gap (Fig 1B, Paper III). There was a similar trend observed in the slow type muscle fibres although the differences in size were not significant (Fig 2B, Paper III).

Characterization of human MSC (Paper V)
MSCs isolated from human bone marrow tissue were negative for haematopoietic markers CD14 and CD19, and positive for mesenchymal stem cell markers CD44, CD54, CD90, CD105 and CD146 (Fig. 1). Human MSC (hMSC) also expressed collagen type IV and fibronectin (Fig. 1). PCR analysis revealed that hMSCs expressed transcripts for nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), insulin like growth factor-1 (IGF-1), vascular endothelial growth factor-A (VEGF-A) and angiopoitein-1 (Fig. 2).

Survival of human MSC in the conduit (Paper IV)
At 3 weeks, in both groups which received daily cyclosporine A (CsA) injections, the biodegradation of the fibrin conduit was already underway in contrast to other

groups, both micro- and macroscopically. PKH26-labelled cells were present within the remnants of conduits both in untreated and cyclosporine A treated groups (Fig. 1).

Macrophage reaction after transplantation of human MSC (Paper IV)

The area of ED1 positive macrophage infiltration was measured in order to investigate the immediate inflammatory response. In rats receiving fibrin conduit filled with fibrin matrix, the area of ED1 staining was 21.5% ± 2.5% (Table 1 and Fig. 2A). Treatment with cyclosporine A did not affect macrophage reaction (Fig. 2B) and transplantation of hMSC increased macrophage infiltration to 41.1% ± 3.4% ($P < 0.001$; Table 1 and Fig. 2C). However, combined hMSC transplantation and CsA injections decreased the area of ED1 staining to 9.9% ± 1.7% ($P < 0.05$; Table 1 and Fig. 2D).

Effects of fibrin conduit with human MSCs on axonal growth (Paper IV)

The distance of regeneration was measured on the longitudinal sections of the conduits from the entry point into the conduit until the furthermost axon stained with β III tubulin and expressed in micrometers (Table 1). The survival time of 3 weeks was chosen to assess early regenerative events at the time when pioneering axons cross a 10 mm gap as has been found in our previous experiments with hollow fibrin glue conduit. In animals receiving the same fibrin conduit but filled with fibrin matrix, regeneration distance was reduced to 2851 ± 176 μm (Fig. 1C). Treatment with cyclosporine A alone or transplantation of human MSC without cyclosporine A injections had no significant effect on regeneration distance (Table 1). In contrast, when transplantation of MSC was combined with cyclosporine A treatment, the regenerating axons continued past the distal end (Fig. 1D) of the harvested specimen (additional 2–3 mm of tissue). Although the distance of axonal growth was greater than the length of the conduit, it was impossible to assess the actual distance of regeneration and the value of 12 mm was used in Table 1. The area occupied by axons stained with βIII tubulin at 3 mm from the proximal nerve stump inserted into the conduit was measured (Table 1). The control value for fibrin conduit filled with fibrin matrix was 23,822 ± 2930 μm^2 (Fig. 1E). Treatment with cyclosporine A significantly increased the area of axonal staining by 254% ($P < 0.001$; Table 1 and Fig. 1F). Surprisingly, transplantation of hMSCs alone did not affect the area of axonal staining (Table 1 and Fig. 1G). However, when hMSCs were combined with cyclosporine A treatment, the area of staining was similar to cyclosporine A treatment alone and significantly increased compared with the control group ($P < 0.001$; Table 1 and Fig. 1H).

Effects of fibrin conduit with human MSC on neuronal regeneration (Paper V)

To assess the regeneration of spinal motoneurons into the distal nerve stump, labelling with a retrograde fluorescent tracer Fluoro Ruby was performed. At 12 weeks after nerve injury and repair with a reversed nerve autograft (NG group in graphs), 1770 ± 73 (mean ± SEM) motoneurons regenerated across the distal graft-nerve interface and extended for at least 10 mm into the distal stump of the sciatic nerve (Table 2, Fig. 3A). After transplantation of fibrin conduit with fibrin matrix, the number of regenerating motoneurons was reduced to 34% when compared with the nerve graft group ($P<0.001$; Table 2, Fig. 3B). Addition of the daily injections of

cyclosporine A or combining the cyclosporine A treatment with transplantation of hMSCs resulted in regeneration of 67% and 64% of spinal motoenurons, respectively ($P > 0.05$; Table 2, Fig. 3C,D). An interesting observation was the difference in appearance of retrogradely labelled dendrites in spinal motoneurons at 12 weeks after Fast Blue application to the medial gastrocnemius nerve (Fig. 3E-H). Following peripheral nerve grafting, second order and third order labelled dendrites were present in many studied motoneurons (Fig. 3E). In contrast, repair of the sciatic nerve with fibrin conduit preserved Fast Blue labelling mainly of the first order dendrites and was followed by appearance of fast Blue labelled microglia-like cells around dendritic arborizations. However, following cyclosporine A treatment alone or cyclosporine A treatment with hMSCs transplantation numerous spinal motoneurons demonstrated both first order and second order Fast Blue-labelled dendritic branches (Fig. 3G,H).

Effects of fibrin conduit with human MSC on muscle recovery (Paper V)

The muscle weights of animals treated with reversed nerve graft recovered to 62% of the contralateral side (Table 2). Fibrin conduit with fibrin matrix reduced the muscle weight ratio to 21% ($P < 0,001$; Table 2) whereas daily cyclosporine A injections alone or in combination with hMSCs improved muscle weight ratio to 54% and 58%, respectively (Table 2). There was no statistically significant difference in muscle weight recovery between the nerve graft, fibrin conduit with daily injections of cyclosporine A and fibrin conduit supplemented with hMSCs and cyclosporine A treatment ($P > 0.05$; Table 2).

The computerized image analysis of muscle fiber size (Fig. 4A) revealed that the mean area of fast type fibres was significantly ($P < 0.05$) diminished in the fibrin conduit repaired group in comparison with nerve graft and cyclosporine A treated groups (Fig. 4A), however there was no statistical difference ($P > 0.05$) between the fibrin conduit group and fibrin conduit with hMSCs and cyclosporine A treatment. Analysis of mean diameter of fast type fibres demonstrated similar changes with significant differences ($P < 0.05$; Fig. 4B) between fibrin conduit and all other experimental groups (Fig. 4B). In contrast, the mean area and diameter occupied by slow type fibres were not significantly different between experimental groups ($P > 0.05$; Fig. 4C,D).

DISCUSSION

BD hydrogel and Schwann cells for peripheral nerve repair (Paper I)

BD hydrogel scaffold is formed through the spontaneous assembly of ionic self-complementary beta-sheet oligopeptides under physiological conditions, producing a hydrogel material (Holmes et al., 2000). The peptides do not elicit any immune response or tissue inflammation when introduced into animals (Ellis-Behnke et al., 2006) and were found to 'knit' the neural tissue. Furthermore, BD hydrogel provides a permissive environment for attachment and differentiation of neural stem cells and allows for synapse formation between cultured neurons (Holmes et al., 2000; Semino et al., 2004; Thonhoff et al., 2008). Similarly, findings from this study showed that BD hydrogel could act as a synthetic extracellular matrix for cultured Schwann cells and provides a suitable substrate for neurite outgrowth from DRG neurons. Our results demonstrate that neonatal Schwann cells significantly improve early axonal regeneration through the conduit. The improvement in the distance of regeneration obtained with transplantation of Schwann cells could be associated with production of a variety of neurotrophic factors including BDNF, GNDF and NT-3 known to stimulate elongation of growing axons (Boyd and Gordon, 2003). Apart from providing a chemotactic influence, Schwann cells form basal lamina and participate in the myelination of regenerating nerve fibres (Ide, 1996; Terenghi, 1999).

BD hydrogel applied as conduit matrix resulted in longitudinal alignment of the growing axons and Schwann cells similar to that normally found in the nerve graft. In contrast, alginate/fibronectin formed clusters of hydrogel trapping the transplanted Schwann cells, simultaneously obstructing the pathway of the growth cone and preventing the transplanted Schwann cells from exerting their beneficial effects on regeneration. Previously, our group observed that following incorporation into the alginate/fibronectin hydrogel, the majority of Schwann cells lose their characteristic shape, display round cell profiles and reduce their metabolic activity (Novikova et al., 2006). Similarly, conduits filled with alginate/fibronectin hydrogel supported limited axonal growth at 3 weeks postoperatively. We found no significant differences between alginate/fibronectin hydrogel alone and alginate/fibronectin supplemented with Schwann cells on axonal regeneration within the conduits.

In long-term experiments, the addition of cultured Schwann cells to either the conduits filled with BD hydrogel or alginate/fibronectin did not improve the neuronal regeneration or recovery of target organs. This could be explained by massive post transplantation cell death as shown earlier in the quantitative studies after cell transplantation into the spinal cord or peripheral nerve (Heine et al., 2004; Hill et al., 2006; Marchesi et al., 2007; McKenzie et al., 2006; Pearse et al., 2007; Shimizu et al., 2007).

Another possible explanation for the lack of sustained improvement at 16 weeks as opposed to promising results at 3 weeks might also include the misdirection of growing axons within the distal nerve stump which could result in a mismatched reinnervation of target organs (IJkema-Paassen et al., 2004) or chronic denervation

of Schwann cells located in the distal nerve stump providing inadequate support for regenerating axons (Sulaiman and Gordon, 2009).

Fibrin conduit for peripheral nerve repair (Papers II & III)

Fibrin is a substrate present after nerve injury and disruption of the blood-nerve barrier which serves as a natural growth terrain extracellular matrix for regenerating axons (Herbert et al., 1998; Williams et al., 1987), attracts leukocytes and macrophages (Kay et al., 1973) and stimulates angiogenesis (Dvorak et al., 1987; Hall, 2007; Petter-Puchner et al., 2007), which are some of the prerequisites for successful regeneration after nerve injury (Terenghi, 1995).

From the conduit design point of view, fibrin glue gel fulfils several criteria of an ideal conduit material (de Ruiter et al., 2009). The mechanical properties of fibrin can be easily modified from unstructured liquid to tubular conduit (Bensaid et al., 2003a; Kalbermatten et al., 2009). Another important feature is the adjustable biodegradability by variations of protease inhibitors and the degree of cross-linkage in the hydrogel (Buchta et al., 2005; Sidelmann et al., 2000; Weisel, 2005) allowing biodegradation matched to the length of the gap and timing of regeneration, thus avoiding problems with potential compression of the nerve (Kalbermatten et al., 2009; Nectow et al., 2012).

Our results indicate that over a 10 mm gap, the hollow fibrin conduit promoted similar recovery of gastrocnemius and soleus muscles to the autologous nerve graft repair. Both treatments induced sprouting in the distal nerve stump; however, implantation of fibrin glue conduit induced proportionally higher sprouting than a nerve graft.

Despite having a comparable effect on recovery of the target organs, counts of motor and sensory neurons demonstrated that fibrin conduit supported regeneration of only 50-60% of neurons when compared with the autologous nerve graft. This indicates that neurons regenerating through the fibrin conduit gave rise to more axonal branches than those of the nerve graft. As it is known that the number of neurons that signal to each motor unit is of importance for precision and coordination of the movements (Simon et al., 2003), regeneration through the fibrin conduit and peripheral nerve graft could lead to the formation of motor units of different sizes and, as a result, promote a different functional recovery.

Fibrin deposited in the sciatic nerve after injury changes the composition of the extracellular matrix and inhibits Schwann cell migration (Akassoglou et al., 2003) and differentiation (Akassoglou et al., 2002). Both at 4 weeks (Kalbermatten et al., 2009) and in the present study at 16 weeks, S100-immunolabelled Schwann cells were found in the conduit together with regenerating axons. It is possible that in contrast to the native Schwann cells and extracellular matrix in the autologous nerve graft, migrating Schwann cells within the fibrin conduit create suboptimal environment for regenerating axons.

The critical peripheral nerve gap in a rat nerve injury model is widely accepted as 10-15 mm (Daly et al., 2012; Nectow et al., 2012), with suboptimal regeneration

obtained for longer gaps (Sinis et al., 2007). Similarly, in this study enlarging the gap had deleterious effects on recovery of target organs. Fibrin conduit resulted in recovery of 81% of the muscle weight of nerve graft control over the 10 mm gap but over the 20 mm gap the muscle reached only 43% of control. On the contrary, in the reverse nerve graft group, similar recovery was noted when the gap was increased from 10 mm to 20 mm. Similar findings were noted over a 10 or 24 mm nerve gap, for type I collagen conduit or human decellularized allograft (Whitlock et al., 2009), with no significant differences between the experimental groups over a 10 mm gap, but repair of a 24 mm gap resulted in a significant deterioration of muscle recovery.

Muscles contain individual fibers, classified broadly depending on their phenotype by the expression of myosin heavy chain proteins and content of oxidative enzyme and mitochondria into slow and fast types. Slow fibres (type I) express high levels of oxidative enzyme whereas fast fibres (type II) have more pronounced glycolytic biochemistry. Some fibres display a combination of the two phenotypes, such as type IIA (Yannas et al., 2007). We have observed a striking difference in gross muscle morphology between animals treated with fibrin conduit and reversed nerve graft over the 20 mm gap. When analyzing the mean area of the fast fibres in these two groups, fibrin conduit had a significantly lower value, with similar trend, but no significant differences for the slow fibres. Following denervation, the muscle phenotype changes as neural stimulation is one of the most important determinants of gene expression in skeletal muscles (Fluck and Hoppeler, 2003), which could in part explain the significant changes in the fast fiber mean area. Considering the similar muscle weights between the reverse nerve graft groups in the 10 and 20 mm nerve gaps and the correlating similarity between mean areas of the fast and slow fibres, nerve graft showed significantly better muscle fiber recovery than fibrin conduit in the long gap. Denervation induced atrophy reduces the area but not the number of the muscle fibres and fast fibres are more sensitive to denervation (d'Albis et al., 1995), which could explain changes we have observed in the gastrocnemius muscle containing mostly type 2 fast fibres.

In conclusion, the novel tubular fibrin conduit resulted in similar effects on axonal sprouting and muscle recovery to the autologous nerve graft over the 10 mm gap. However, when compared with autologous nerve graft in the 20 mm gap, fibrin conduit showed significantly worse outcome for muscle recovery. This further supports the need for modifications of the biosynthetic scaffold with cultured glial or stem cells, exogenous growth factors and extracellular matrix molecules such as laminin and fibronectin (Boyd and Gordon, 2003; Kannan et al., 2005; Pfister et al., 2007; Yannas et al., 2007), which could potentially improve neuronal regeneration through the conduit.

Fibrin conduit with human MSC for peripheral nerve repair (Papers IV & V)

Similarly to previous reports from our research group (Brohlin et al., 2009) and others (Prockop et al., 2001) the results of this thesis demonstrated that hMSCs express typical mesenchymal cell markers in addition to neurotrophic factors such as BDNF, NT-3, GDNF, NGF and VEGF (Brohlin et al., 2009; Ghorbanian et al., 2012; Himes et al., 2006). It is well known that these neurotrophic factors can enhance survival of sensory dorsal root ganglion neurons and spinal motoneurons

(Ljungberg et al., 1999; Novikov et al., 1995) and promote axonal regeneration after peripheral nerve injury (Gordon, 2009; Madduri and Gander, 2010).

In the present experimental settings, it was difficult to demonstrate any potential beneficial effects of hMSCs transplantation due to significant effects of daily cyclosporine A injections. Our results indicated that although cyclosporine A increased axonal sprouting in the proximal part of the conduit, regeneration distance was relatively short and did not differ from the group with MSCs without immunosupression. In contrast, combining MSCs transplantation with cyclosporine A treatment resulted in a significant enhancement of axonal growth. The low impact of the hMSCs on axonal regeneration could be due to increased cell death in the present xenogenic model when compared with transplantation of rat MSCs (di Summa et al., 2011). Similarly, encouraging results have been seen with the application of human bone marrow-derived or umbilical cord-derived mesenchymal stem cells differentiated into glial lineage (Matsuse et al., 2010; Shimizu et al., 2007), in combination with the FK506. However, these studies lack a control group without immunosuppression. In the studies where hMSCs have been transplanted in immunologically competent rats without immunosuppresion and favourable results of treatment have been found, the gap between nerve ends was much shorter than in our experiment and no morphological analysis was performed making the studies difficult to compare (Pan et al., 2006). No statistical difference in ED1 positive macrophage infiltration in groups without cell transplants was observed in the study. The highest number of macrophages was found in a group where human MSC were transplanted without cyclosporine A treatment. Fibrin glue was found to attract macrophages (Ussia et al., 1998) which can possibly explain this finding as we have observed non- degraded fibrin glue present without cyclosporine A treatment. The addition of cyclosporine A resulted in faster biodegradation of fibrin with less macrophages as a reaction to transplanted human mesenchymal stem cells as shown with ED1 staining.

It has been also shown that cyclosporine A stabilizes mitochondrial membrane potential, up-regulates Bcl-2 and down-regulates Bax expression (Wang et al., 2008). As a result, several studies demonstrate that cyclosporine A can support the survival of transplanted fibroblasts (Hayashi et al., 2005), mesenchymal stem cells and adult neural precursor cells (Parr et al., 2008). However, there are also reports that conventional immunosuppressive treatment with cyclosporine A is not sufficient to prevent death of hMSCs after transplantation in long-term experiments (Himes et al., 2006; Khoo et al., 2011). Although the primary target cells of cyclosporine A are T-lymphocytes, several functions of macrophages are affected both *in vitro* (chemotaxis, interleukin-1 generation and prostaglandin E production) and *in vivo* (decreased macrophage infiltration observed in animals treated with 20 mg/kg of cyclosporine A but not at lower doses) (Matsushima and Baba, 1990). Cyclosporine A regulates also expression of matrix metalloproteinases (MMP) (Bianchi et al., 2003), which in turn could have accelerated the biodegradation of fibrin glue as seen in our study (Bini et al., 1996).

Another explanation is that cyclosporine A has a significant neuroprotective effect on its own (Ibarra and Diaz-Ruiz, 2006) and promotes neuroregeneration and

neuronal extension via induction of GAP-43 (Strittmatter et al., 1992). Therefore cyclosporine A treatment can "mask" the potential neuroprotective effects of hMSCs transplantation. Thus, in the present study cyclosporine A treatment significantly improved recovery of gastrocnemius muscle weight and morphology of fast type muscle fibers. Treatment with cyclosporine A could be omitted if human cells are transplanted in the nervous system of athymic nude rats. A recent report has demonstrated that human mesenchymal precursor cells (Stro-1^{+}) transplanted into the injured spinal cord of nude rats can promote significant functional recovery (Hodgetts et al., 2012).

The mechanisms of the neuroprotective effect of cyclosporine A could be due to down-regulation of nitric oxide, a well known neurotoxic agent (Ibarra and Diaz-Ruiz, 2006) and direct inhibition of calcineurin, a potent regulator of muscle remodeling (Hui et al., 2010). Recently, cyclosporine A has been considered a neuroprotective agent for the treatment of acute traumatic brain injury in patients (Cook et al., 2009) since it can interrupt the endogenous mediators of secondary insult through inhibition of the mitochondrial permeability transition pore and prevention of subsequent mitochondrial dysfunction (Kilbaugh et al., 2011; Signoretti et al., 2004; Sullivan et al., 2005; Toman and Fiskum, 2011). Previous studies also show that cyclosporine A can support axonal regeneration after spinal cord injury (Hayashi et al., 2005; Ibarra et al., 2007), increase the regrowth of the retinal ganglion cells into the peripheral nerve graft (Gillon et al., 2003) and accelerate the peripheral nerve regeneration (Jost et al., 2000). In agreement with these observations, our previous study demonstrates that cyclosporine A treatment can induce significant axonal sprouting inside a fibrin conduit (McGrath et al., 2012).

However, long-term cyclosporine A treatment could have unwanted side effects such as weight loss and muscle weakness, possibly due to inhibition of calcineurin activity (Sakuma and Yamaguchi, 2010). Although other studies have suggested that calcineurin inhibition by cyclosporine A modulates muscle phenotype rather than muscle mass (Aoki et al., 2006) and results in an increase of type IIa MHC (fast fiber type) content at the expense of type I MHC (slow fiber type) (Zbreski et al., 2006). Analysis of muscle morphology in the present study also revealed that cyclosporine A has a positive effect on restoration of fast fiber type area and diameter. With respect to slow type muscle fibers, the mean area and diameter were not statistically different between experimental groups and this observation is in agreement with a previous report that in denervated muscle a slow phenotype is triggered and maintained in a calcineurin- and nerve-independent manner (Launay et al., 2006).

The future advances in the field of peripheral nerve regeneration are likely to be based on even more pronounced convergence of the academic and clinical environments. The progress in treatment of peripheral nerve injuries occurs currently mainly in the laboratories (Cunha et al., 2011), however, validation of experimental treatment strategies in the clinical settings determines the directions of future research.

A multilevel approach, based on the manipulation of several factors influencing the outcome of nerve regeneration would focus on neuroprotection (Hart et al., 2008), early exploration and nerve repair to avoid deleterious effects of the delay (Jivan et al., 2006), optimising cell-based therapy for gap management (Walsh and Midha, 2009a) and choice of biomaterials (Daly et al., 2012; Nectow et al., 2012; Subramanian et al., 2009), introducing new strategies to avoid denervation of Schwann (Sulaiman and Gordon, 2009) and affecting the hostile milieu in the distal stump to preserve the target organs while awaiting reinnervation. There is a plethora of new and revolutionary ideas such as avoiding tubular conduit for the gap repair (Kalbermatten et al., 2008a), gene manipulations to induce the neurons to the state found during development of peripheral nervous system or during limb regeneration found among certain vertebrates (Suzuki et al., 2005) and avoiding Wallerian degeneration all together with immediate splicing of injured axonal membranes (Chang et al., 2009) resulting hopefully in restoration of axonal transport and activity. Hopefully, in the future, the goalposts of what the researchers and clinicians are trying to achieve will move from working towards a replacement for a nerve graft with its suboptimal outcome to aiming towards restoring original function of the injured nerves.

CONCLUSIONS

This thesis investigates the effects of a synthetic matrix BD™ PuraMatrix™ peptide hydrogel, alginate/fibronectin hydrogel and fibrin conduit combined with cultured rat Schwann cells and human mesenchymal stem cells (MSC) on neuronal regeneration and muscle recovery after peripheral nerve injury in adult rats.

The following conclusions were made based on the experimental data:

- The addition of neonatal Schwann cells to the BD hydrogel considerably increases axonal regeneration distance at 3 weeks postoperatively but failed to extend this effect on long-term regeneration of spinal motoneurons and muscle recovery.

- The fibrin conduit and reversed nerve graft have similar effects on the sprouting of myelinated axons in the distal nerve stump and muscle recovery in a 10 mm gap. The fibrin conduit demonstrated significantly inferior muscle recovery when compared with the peripheral nerve graft in a 20 mm gap.

- The fibrin conduit seeded with human MSC enhanced axonal regeneration 3 weeks after sciatic nerve injury and repair only when transplantation was combined with cyclosporine A treatment. Addition of cyclosporine A treatment also improved human MSC survival and significantly reduced the macrophage reaction.

- Repair of the injured sciatic nerve with fibrin conduit and cyclosporine A treatment induced restoration of the muscle weight and the size of fast type fibers to the control levels of the nerve grafting group in the long-term experiments. The addition of human MSC did not further improved muscle recovery.

- The results indicate that although human MSC expressed growth-promoting factors necessary for successful nerve regeneration, the neuroprotective effect of cyclosporine A treatment can “mask” the potential beneficial effects of human MSC transplantation.

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