UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
New Series No. 1369 ISSN 0346-6612 ISBN 978-91-7459-038-8

NERVE GAP REPAIR
by the use of artificial conduits and cultured cells

Daniel Felix Kalbermatten

Department of Integrative Medical Biology
Section for Anatomy
Department of Surgical and Perioperative Sciences
Section for Hand & Plastic Surgery
Umeå University, Sweden, 2010

Responsible publisher under Swedish law: the Dean of the Medical faculty

ISBN: 978-91-7459-038-8
ISSN: 0346-6612
Printed by: Print and Media, Umeå, Sweden 2010

ABSTRACT

Peripheral nerve injuries are often associated with loss of nerve tissue and require autologous nerve grafts to provide a physical substrate for axonal growth. This thesis investigates the use of fibrin as both a tubular conduit to guide nerve regeneration and also as a matrix material to suspend various regenerative cell types within/on poly-3-hydroxybutyrate (PHB) nerve conduits. Adipose derived stem cells (ASC) are found in abundant quantities. In this thesis the ability of rat ASC to differentiate into Schwann cells was determined and a preliminary study of the neurotrophic potential of human ASC was also investigated.

Rat sciatic nerve axotomy was performed proximally in the thigh to create a 10-mm gap between the nerve stumps and the gap was bridged using the various conduits. At early time points the nerve grafts were harvested and investigated for axonal and Schwann cell markers. After 16 weeks the regenerative response from sensory and motor neurons was also evaluated following retrograde labelling with Fast Blue fluorescent tracer. Stem cells were treated with a mixture of glial growth factors and after 2 weeks *in vitro* the expression of Schwann cell markers was analysed by immunocytochemistry and Western blotting. ASC were cocultured with the NG108-15 neuronal cell line to determine their ability to promote neurite outgrowth. Human ASC were isolated from the deep and superficial layers of abdominal fat tissue obtained during abdominoplasty procedures. RT-PCR was used to investigate the expression of neurotrophic factors.

Immunohistochemistry showed a superior nerve regeneration distance in the fibrin conduit compared with PHB. The fibrin conduit promoted regeneration of 60% of sensory neurones and 52% of motor neurones when compared with an autograft group at 16 weeks. The total number of myelinated axons in the distal nerve stump in the fibrin-conduit group reached 86% of the graft and the weight of gastrocnemius and soleus muscles recovered to 82% and 89% of the controls, respectively. *In vitro* studies showed that rat ASC could be differentiated to a Schwann cell phenotype. These treated cells enhanced both the number of NG108-15 cells expressing neurites and neurite length. In the same coculture model system, human superficial fat layer ASC induced significantly enhanced neurite outgrowth when compared with the deep layer fat cells. RT-PCR analysis showed ASC isolated from both layers expressed neurotrophic factors.

These results indicate that a tubular fibrin conduit can be used to promote neuronal regeneration following peripheral nerve injury. There was also a beneficial effect of using a fibrin matrix to seed cells within/on PHB conduits which should ultimately lead to improved functional recovery following nerve injury. There might also be an advantage to use a simple strip of PHB rather than a conventional tube-like structure implying that single fascicle nerve grafting could be advantageous for nerve repair. The results of *in vitro* experiments indicate adipose tissue contains a pool of regenerative stem cells which can be differentiated to a Schwann cell phenotype and given that human ASC express a range of neurotrophic factors they are likely to be of clinical benefit for treatment of peripheral nerve injuries.

Keywords: adipose stem cells, cell matrix, fibrin, nerve conduit, nerve gap

ORIGINAL PAPERS

This thesis is based on the following papers which are referenced in the text with Roman Numerals.

I
Kalbermatten DF, Pettersson J, Kingham PJ, Wiberg M, Pierer G, Terenghi G: New Fibrin Conduit for Peripheral Nerve Repair. Journal of Reconstructive Microsurgery (2009) 25, 1: 27-33.

II
Pettersson J, Kalbermatten DF, McGrath A, Novikova LN: Biodegradable fibrin conduit promotes long-term regeneration after peripheral nerve injury in adult rats. Journal of Plastic, Reconstructive & Aesthetic Surgery (ahead print 2010)

III
Kalbermatten DF, Kingham PJ, Mahay D, Mantovani C, Pettersson J, Balcin H, Pierer G, Terenghi G: Fibrin matrix for suspension of regenerative cells in an artificial nerve conduit. Journal of Plastic, Reconstructive & Aesthetic Surgery (2008) 61: 669-675.

IV
Kalbermatten DF, Erba P, Mahay D, Wiberg M, Pierer G, Terenghi G: Schwann cell strip for peripheral nerve repair. The Journal of Hand Surgery (European Volume, 2008) 33 5: 587–594.

V
Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G: Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth *in vitro*. Experimental Neurology (2007) 207: 267–274.

VI
Kalbermatten DF, Schaakxs D, Kingham PJ, Wiberg M: Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. Manuscript

Abbreviations

ASC	adipose derived stem cell
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMSC	bone marrow stem cell
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindol
DMEM	Dulbecco's medium
DRG	dorsal root ganglia
dASC	differentiated adipose derived stem cell
dMSC	differentiated mesenchymal stem cell
FBS	foetal bovine serum
GFAP	glial fibrilliary acidic protein
GGF	glial growth factor
GFP	green fluorescent protein
MSC	mesenchymal stem cell
NGF	nerve growth factor
NTF	neurotrophic factor
PDGF	platelet derived growth factor
PGP9.5	protein gene product 9.5
PHB	poly-3-hydroxybutyrate
PNS	peripheral nervous system
SC	Schwann cell
uASC	undifferentiated adipose derived stem cell
uMSC	undifferentiated mesenchymal stem cell

INTRODUCTION

Clinical background and classification of nerve injuries

Most commonly nerve lesions are caused by transection, compression, crush, traction and avulsion mechanisms. Three main groups help to classify the lesions: neurapraxia, axonotmesis and neurotmesis. Neurapraxia is a transient condition presenting a physically intact nerve. If axons and myelin sheath are damaged the lesion is classified as axonotmesis which can regenerate spontaneously (Seddon et al. 1943). However neurotmesis presents a disruption of the axon and the myelin sheath which does not recover without surgical repair. Sunderland refined this classification further, suggesting five degrees of severity (Sunderland 1951; Sunderland 1965; Sunderland 1990) verified by using histological analyses.

Each year there are about 300,000 peripheral nerve injuries in Europe, a considerable number of them involving the nerves of the hand and arm, and despite advances in microsurgical techniques the functional results are poor for the patient. Division of a peripheral nerve results in impaired sensation, reduced motor function and sometimes pain. Such injuries have a profound and permanent impact of the patient's life as they do not regain normal function. There are also serious economic implications for both the individual patient and society, and the average period of rehabilitation required and the mean time off work ranges from 21.4 to 31.3 weeks, with just 59 – 69% of patients back in full time work 1 year after injury. The total expense per patient is estimated to be EUR 51,238 for median and ulnar nerve injuries (Rosberg et al. 2005a; Rosberg et al. 2005b).

Nerve injuries without defects (loss of nerve tissue) or with a short gap are usually treated by an end-to-end coaptation (epineural suturing). The normal nerve segments, proximal and distal to the site of neurorrhaphy are sufficiently extensible to compensate for the short defects. If there is a longer defect, a neurorrhaphy without tension at the site of the repair cannot be performed (Millesi 2006) and surgical repair of nerve gaps greater than 20 mm is

commonly achieved by autologous nerve grafts. Thus neural injuries represent a major clinical challenge and in particular when they are accompanied by a loss of neural tissue leading to gaps or cavities. In the peripheral nervous system, a loss of nerve material necessitates the bridging of the gap using autologous nerve grafts most commonly taken from the patients legs, resulting in morbidity at the donor site (loss of sensation, scarring, pain).

Biology of peripheral nerve degeneration and regeneration

Peripheral nerves can to some extent regenerate after an injury unlike the limited regeneration of nerves in the central nervous system (CNS). If a peripheral nerve is injured it triggers a series of events resulting in activation of Schwann cells (SCs) and macrophages (Burnett and Zager 2004). The regenerative ability of peripheral nerves is attributed to the interactions between these cellular elements and the extracellular matrix molecules of the SC basal lamina (McAllister and Calder 1995; Hall 1997). The degeneration process is described as Wallerian degeneration and occurs at the distal end of the stump. The proximal nerve also shows degeneration retrogradely up to the nearest node of Ranvier. Macrophages are responsible for phagocytosis of degraded components and contribute to the transient proliferation of SCs which align to form the Schwann cell columns or “bands of Büngner”. The SC and basal lamina scaffold acts as guidance, providing an environment for regenerating axons in the proximal nerve stump to grow across the lesion (Ide 1996; Hall 1997). Many intracellular alterations happen at the site of injury which regulate the following regeneration of the axons and the reconstitution of the neuronal cytoskeleton (Schlaepfer and Bunge 1973).

Two populations of macrophages are involved in the degradation at the injury site; the resident and migrating blood borne macrophages. Logically the resident cells respond immediately to the injury whereas the recruited cells arrive in the second phase of healing after 72 hours (Mueller et al. 2001). Inflammatory mediators are released from macrophages including interleukin

1β (IL-1β) and tumour necrosis factor alpha (TNF-α) as well as IL-α secretion from activated SCs (Heumann et al. 1987; Lindholm et al. 1987; Be'eri et al. 1998; Shamash et al. 2002). However, SC are the most important cells in the regeneration process and release neurotropic factors that diffuse proximal to the lesion site and stimulate a vast amount of sprouting axons that also can be modulated in a dynamic process of out sprouting and regression (Brushart 1993). SC can also upregulate myelin genes and synthesise new myelin to form functional nerve fibres (Gupta et al. 1993).

Schwann cell biology

SCs help axons to regenerate as they release neurotrophic factors (NTFs) after an injury. SCs also have a phagocytic function and help to eradicate degenerated structures from necrotic nerves. Many studies have found their important role as ideal therapeutic cell target to heal nerve injuries. Nerve grafts seeded with SCs have shown benefits to axon regeneration (Guenard et al. 1992). Addition of SC also leads to a better long gap regeneration (Bryan et al. 1996) and enhanced functional recovery (Ansselin et al. 1997). SCs release NTFs such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) after an injury. NTFs are polypeptides which ensure survival and differentiation of neurons and regulate growth of neurites. Release of NTFs is mediated in target tissues for uptake by intact nerve fibres. (Ide 1996). Although SCs provide a better regeneration (Mosahebi et al. 2001) they are not an ideal transplantable cell because they are not easy accessible and slow to grow *in vitro* (Tohill et al. 2004). Culturing a vast amount of cells requires a long time and needs constant growth factor supply. Therefore these cells only could be used for delayed repair which is known to compromise axonal regeneration (Sulaiman and Gordon 2000). In addition, a nerve biopsy is required to isolate cells and therefore results in some donor site morbidity.

Neural injury repair strategies

An ideal substitute to treat a nerve gap has not been found. An autologous nerve graft provides SCs, growth factors and basal lamina components and is

the current gold standard but has associated problems; scarring, neuroma formation and loss of sensation at the donor site are common consequences. In addition, when looking at general functional recovery, previous studies have also reported a poor recovery of sensation as well as only partially recovered motor function in most cases (Young et al. 1980; Dellon and Jabaley 1982; Beazley et al. 1984; Mackinnon and Dellon 1988). Nerve autograft alternatives have been sought and include autologous conduits such as venous or arterial conduit grafts but these have not demonstrated any functional benefits compared to standard nerve grafts (Keskin et al. 2004; Battiston et al. 2005). Peripheral nerve allografts using cadaveric tissue have been tested, but they have many limitations especially because of the undesirable long-term immunosuppressive therapy required (Evans et al. 1994). In order to achieve a better clinical outcome, several synthetic conduits have been studied to replace nerve autografts and allografts. Non-degradable materials such as silicone (Mackinnon and Dellon 1988; Merle et al. 1989; Lundborg 1991), polytetrafluorethylene (PTFE) and polypyrrole (PPY) have been thought to provide a permissive environment for outgrowing axons allowing the supportive supply of neurotrophic factors and SCs (Gibson et al. 1991) . However, it has been noted that compression syndromes often occur because of their non-degradable nature and their inability to adapt to the nerve growth and maturation (Mohanna et al. 2005). Moreover, increased scarring and irritation of the patient has been described (Albala and Lawson 2006). Most common at present, synthetic conduits used for bridging neural gaps are made of biodegradable or bioresorbable materials (Le Guehennec et al. 2004). Among these, poly 3-hydroxybutyrate (PHB) conduits have gained particular interest because of their soft malleable consistency, good tensile strength and flexibility. PHB conduits show early vascularisation after implantation and are resorbed over a period of two years. Nevertheless these conduits have a rather long resorption time and ideally an optimal conduit would dissolve faster, having supported the sprouting axons and allowing neurotrophic factors to penetrate during the early phase of regeneration.

Table 1. History of conduits in nerve gap repair.

1979	Silicone	Lundborg G
1980	Synovialis	Lundborg G
1983	Muscle	Ide
1988	PGA (Polyglycol)	Dellon
1988	Vein	Chiu
1990	Carbonate	Mackinnon
1993	Collagen	Kim
1999	Alginate	Suzuki
2000	Suture	Scherman&Lundborg
2002	Tendon	Brandt&Lundborg
2002	PHB (Poly-3-hydroxybutyrate)	Young,Terenghi,Wiberg

One of the aims of this thesis is to evaluate the use of fibrin as a conduit to guide nerve regeneration and bridge nerve defects. The use of fibrin, a naturally occurring molecule, mimics the physiological properties when a nerve is healing. Fibrin glue is used widely in surgical practice (Fang et al. 2004). It helps surgeons to adapt tissues and to obtain haemostasis in difficult situations. It has also been used in coaptation of nerves with good results (Ornelas et al. 2006). Fibrin glue can be fabricated autologously from individual donors or is commercially available from different companies for clinical use (Le Guehennec et al. 2004) . Fibrin glue can be diluted specifically to change its dissolving and coagulations characteristics (Bensaid et al. 2003). Fibrin glue also has good biocompatibility and has been used as a scaffold material in bone tissue engineering (Albala and Lawson 2006). One of the objectives of this work is to investigate whether fibrin supports the growth and differentiation of SCs and other important regenerative cell types.

Role of stem cells in neural repair

In recent years the field of stem cell therapy has expanded rapidly. The use of embryonic stem cells, however, remains controversial and their clinical application is likely to be limited due to ethical, legal and political

considerations. Adult stem cells, such as those from bone marrow and adipose tissue, represent a more readily acceptable source of cells. At present, mesenchymal stem cells (MSC) isolated from the bone marrow are the best characterised and will respond to a variety of chemical agents and growth factors to generate a number of mature phenotypes, along committed cell lineages (Bianco et al. 1999). Experiments have shown that it is possible to induce bone marrow MSC to adopt a Schwann cell phenotype (Caddick et al. 2006) which, when transplanted into nerve conduits, enhance peripheral nerve regeneration (Tohill and Terenghi 2004). However, donor site morbidity limits the amount of bone marrow that can be obtained and thus extends the time in culture required to generate a sufficient number of cells for therapeutic use.

Other more easily accessible sources of adult stem cells have therefore been sought. Recent studies indicate that adipose tissue might also contain nascent stem cells. Fat can be digested enzymatically and the stem cell population pelleted by centrifugation and easily isolated from the mature adipocytes which float in the supernatant. Furthermore, lipoaspirate obtained following cosmetic procedures has been shown to be an excellent starting material for isolation of these cells (Gimble et al. 2007). The significance of this for stem cell therapy is clear. For instance, 40 ml of bone marrow (the maximum normally extracted) contains approximately 20000 stem cells whilst 200ml of lipoaspirate (a routine quantity extracted) contains over 1 million stem cells (Strem et al. 2005). Adipose-derived stem cells (ASC) can differentiate along classical mesenchymal lineages resulting in the formation of fat, bone and cartilage tissue as well as neuronal cells (Strem et al. 2005).

AIMS OF THE STUDY

The overall aim of this project was to investigate fibrin as a nerve conduit and matrix material in combination with different regenerative cells for peripheral nerve gap repair.

Specific objectives:

- To investigate the effects of a tubular fibrin glue conduit on axonal regeneration after peripheral nerve injury.
- To assess the effect of fibrin as a matrix material to transplant Schwann cells or bone marrow stem cells for peripheral nerve repair.
- To compare the growth-promoting effects of PHB strip and PHB tubular conduit on peripheral nerve regeneration using fibrin to seed cells.
- To determine the neuroregenerative potential of adipose stem cells (ASC) *in vitro*.
- To evaluate an ideal donor site for ASC harvesting in humans.

MATERIALS AND METHODS

ANIMALS

All short term (2-4 week survival) experiments were performed on adult female Sprague-Dawley rats (Harlan, UK). For the long term (16 week survival) studies adult female Fisher rats (Scanbur, SE) were used. Studies were approved by the Northern Swedish Regional Committee for Ethics in Animal Experiments (Nr. A9-08). The animal care and experimental procedures were carried out in accordance with the standards established by the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985) and the European Communities Council Directive (86/609/EEC). All surgical procedures were performed under general anesthesia with iterated intra-peritoneal injections using a mixture of ketamine (Ketalar, Parke-Davis) and xylazine (Rompun, Bayer). Benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.) was given after each surgical procedure. Isolation of cells from rats was performed following euthanasia by CO_2 according to the local veterinary commission University of Lausanne and carried out in accordance with the European Community Council directive 86/609/ECC for the care and use of laboratory animals.

CELL CULTURE

Schwann cells (SCs) - Papers III,IV,V

To harvest SCs, sciatic nerves were exposed, removed and kept in Dulbecco's Modified Eagle's Medium plus Glutamax containing 1% penicillin-Streptomycin (DMEM, Invitrogen, UK). Nerves were then dissected in trunks, desheathed and finally cut in 1mm segments under a microscope. The segments were then plated in a petri dish with growth medium supplemented by 14 μM forskolin and 40 ng/ml GGF-2 SC growth medium. Segments were incubated for 2 weeks at 37°C with 5% CO_2 and fresh medium was added approximately every 72 h. After 2 weeks, medium was aspirated and 0.125% collagenase type IV and 117U/mg dispase were added to the petri dish. After 24h incubation the cell suspension was filtered through a 70μm cell strainer (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and centrifuged at 900 rpm for 5

min to obtain the cell pellet. Finally the cell pellet was resuspended in SC growth medium, seeded into 25cm^2 flask pre-coated with Poly-D-Lysine (Sigma, St Louis, MO) and incubated in the same conditions. The following day the medium was replaced and cells were left to proliferate. At confluence, the SCs were purified by an antibody complement method to eradicate remaining fibroblasts by initially resuspending the SCs in mouse anti-rat Thy 1.1 antibody (1:500, Serotec, UK) Rabbit complement was then added to the cells, followed by centrifugation at 600g for 5 min. The supernatant was aspirated and the cell pellet suspended in SC growth medium. Cells were then plated and incubated in 5% CO2.

Mesenchymal stem cells (MSC) - Papers III,IV,V

Mesenchymal stem cells were harvested from adult Sprague Dawley rat femoral bones (Tohill et al. 2004): mesenchymal cell growth medium (α-MEM; Invitrogen; supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin) was injected through each marrow cavity using a 21G needle. The resulting cell suspension was triturated, filtered through a 70 μm Falcon filter and centrifuged for 5 min at 600g. The supernatant was aspirated, the cell bolus resuspended in mesenchymal cell growth medium, and the cells plated in 75 cm^2 tissue culture flasks and incubated in 5% CO_2 at 37°C. Haematopoietic cells were eliminated by washing daily with DMEM until all non-adherent cells were removed. The cells were then allowed to grow to confluence.

Adipose derived stem cells (ASC) - Papers V and VI

After euthanasia, omental or inguinal fat pad tissue was carefully dissected and minced using a sterile razor blade. Tissue was enzymatically digested for 120 min at 37°C using 0.15% (w/v) collagenase type I (Invitrogen, UK). The solution was passed through a 70-μm filter to remove undissociated tissue, neutralized by the addition of α-MEM containing 10% (v/v) foetal bovine serum (FBS) and centrifuged at 800×g for 5 min. The stromal cell pellet was resuspended in growth medium containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cultures were maintained at sub-confluent

levels in a 37 °C incubator with 5% CO_2 and passaged with trypsin/EDTA (Invitrogen, UK) when required. The same protocol was used for isolation of human ASC. One gram of adipose tissue from the two abdominal fat layers (superficial and deep) was isolated during the abdominoplasty procedure on 20 patients. The study followed the ethical guidelines of the Swiss Academy of Medical Sciences using informed consent management.

Rat stem cell differentiation - Papers III,IV,V

Undifferentiated ASC and MSC where differentiated at early passages. Growth medium was removed from sub-confluent cultures and replaced with medium supplemented with 1 mM β-mercaptoethanol (Sigma-Aldrich, UK) for 24 h. Cells were then washed and fresh medium supplemented with 35 ng/ml all-trans-retinoic acid was added. A further 72 h later, cells were washed and medium replaced with differentiation medium; cell growth medium supplemented with 5 ng/ml platelet-derived growth factor (PDGF; PeproTech Ltd., UK), 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech Ltd., UK), 14 μM forskolin and 252 ng/ml GGF-2. Cells were incubated for 2 weeks under these conditions with fresh medium added approximately every 72 h.

PREPARATION OF NERVE CONDUITS FOR SURGERY

Poly-3-hydroxybutyrate (PHB) tubular conduit and strip - Papers I-IV

PHB (Astra Tech AB, Mölndal, Sweden) sheets were used to form the conduits by rolling around an intravenous cannula (16G Abbocath®, Abbott, Ireland) and heat-sealed to form a conduit (14mm long and 2mm diameter). Alternatively PHB sheets were cut to create a strip (14mm x 0.5mm).

Fibrin conduit - Papers I,II

The fibrin conduit was prepared from two compound fibrin glue (Tisseel® Kit VH 1.0, Baxter SA, USA). Tisseel® contains fibrinogen, 70-110 mg/mL; plasma fibronectin, 2-9 mg/mL; factor XIII, 10-50 U/mL; plasminogen, 40-120 μg/mL, aprotinin solution 3000 KIU/ml, thrombin 4IU/mL, calcium chlorides 40 mmol/L. Fibrin glue was dispensed in a silicone prepared mold around a

stainless steel core and pressed into shape for five minutes. This allowed the generation of uniform conduits measuring 14mm in length, with a 2mm lumen and 1 mm wall thickness.

Cell suspension in fibrin matrix - Papers III, IV

A fibrin matrix was constructed by diluting the two compound fibrin glue (Tisseel® Kit VH 1.0, Baxter SA, Switzerland). Thrombin was diluted 1/100 and fibrinogen 1/10. Buffers for the dilutions were used according to the group of Bensaid and consisted of 10 ml de-ionized water enriched with 73.5 mg sodium citrate, 16.9mg sodium chloride, 249.9 mg glycine, 30'000UIK aprotinin (Sigma, A-3428, Sigma-Aldrich, USA) and 150mg albumin serum (Sigma, A-8763, Sigma-Aldrich, USA). A solution of 10ml with de-ionized water was made to the dilution of the thrombin containing 58.8mg calcium chloride, 87.1mg sodium chloride, 30.3mg glycine and 500mg serum albumin. Using this solution it was possible to dilute the two components accordingly, generating the previously published fibrin gel to thrombin ratio 9/50 (mg/IU). Flasks of confluent cells were trypsinised and cells pelleted to mix with the diluted fibrinogen in a microtube. For the control group the cells were mixed with growth medium without diluted fibrinogen. This procedure created a cell-fibrinogen suspension that clots when it is placed in contact with plated thrombin. Such suspensions were used in experiments testing cell attachment properties on tissue culture plastic surfaces and PHB material. $80x10^6$ cells/ml were suspended in 25µl of fibrinogen solution. PHB strips and conduits were coated with 25µl of the diluted thrombin (5 IU/ml) solution for 10 min and then fibrinogen/cell solution was added. The two components clot and produce fibrin enabling the cells to be uniformly distributed.

SURGICAL PROCEDURE AND EXPERIMENTAL GROUPS

A sciatic nerve lesion creating a 10mm gap was used. The left sciatic nerve was approached dorsally using a gently spreading technique of the gluteus muscle. Once identified, the nerve was divided with microsurgical scissors at a distance of 10mm from its emergence of the sacral notch. After resection of the nerve it

was either used as a reverse autograft or was replaced by an artificial conduit or a strip fabricated from different materials and cells (see Table 1 below). Surgical implantation of the structures was undertaken with an operating microscope (Zeiss, Germany) and the nerve ends were fixed to the conduit/graft by a single epineural suture (9/0 Prolen, Ethicon): proximal and distal nerve stumps were inserted 2 mm into the nerve conduits thus leaving a 10 mm gap. Muscles and fascia layers were closed with single resorbable stitches (4/0 Softcat, Braun) and the skin by using a continuous running suture (4/0 Prolen, Ethicon).

Table 1.

Papers I-IV	Species	Groups (6 each group)	Total n
Fibrin Conduit short term	SD (Harlan)	2	12
Fibrin Conduit long term	Fisher (Scanbur)	2	12
Fibrin Matrix	SD (Harlan)	4	24
Schwann Cell Strip	SD (Harlan)	2	12

EX VIVO TISSUE HARVEST AND PROCESSING

Nerve conduit harvesting and immunohistochemistry - Papers I-IV

Animals were sacrificed after 2 or 16 weeks. Harvested conduits were fixed by paraformaldehyde (PFA) for 16 h, then rinsed 24 h at 4°C in PBS containing 30% sucrose and 0.1% sodium azide, with PBS changes after 12 h to eliminate any PFA remnants. The specimens were then embedded in OCT freezing media (Tissue-tek, Sakura, Japan) and longitudinal cryo-sections (14 μm) were prepared onto slides (Superfrost® plus, Menzel-Gläser, Germany). After 3 x 5 min PBS washes, either an appropriate normal serum or a casein solution was applied for 1h to block nonspecific antibody binding. Slides were incubated either for 2h at room temperature or overnight at 4°C with primary antibodies rabbit anti-S100 (1:500, Dako, UK), rabbit anti-PGP 9.5 (1:500, Dako, UK) or βIII tubulin (1:1000, Sigma-Aldrich). Subsequently slides were washed 3 x 5 min in PBS and were then incubated with secondary goat anti-rabbit and goat anti-mouse antibodies Alexa Fluor 568 and Alexa Fluor 488 (1:500 and 1:300;

Invitrogen, USA) at room temperature in the dark for 40 minutes. After 3 x 5min PBS washes, the slides were finally mounted with Vectashield with DAPI (Vector Labs, UK) and examined under the fluorescence microscope (10x magnification, Olympus IX81). The staining specificity was tested by omission of primary antibodies. Axonal regeneration distance (PGP 9.5, βIII tubulin) and S100 positive cell distribution inside the conduit were evaluated.

Retrograde neuronal labelling - Paper II

In order to identify sensory dorsal root ganglion (DRG) neurons and spinal motor neurons which had regenerated through the neural conduit, the tibial nerve was identified and transected 10 mm from the distal graft end at 15 weeks after transplantation. A small polyethylene tube was heat-sealed from one end, filled with 2-3µl of 2% aqueous solution of fluorescent tracer Fast Blue (FB, EMS-Chemie GmbH, Germany) and fixed to the surrounding tissues with Histoacryl® glue (B.Braun Surgical GmbH, Germany). The proximal stump of the tibial nerve was placed into the tube and the open end of the tube was sealed with a mixture of silicone grease and Vaseline to prevent leakage. Two hours later the cup with 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 termination of the experiment.

Tissue processing after retrograde labelling - Paper II

At the end of the survival period of 16 weeks, the animals were transcardially perfused with Tyrode's solution (37°C) followed by cold 4% paraformaldehyde (pH=7.4). The spinal cord segments L4–L6 and homonymous DRGs were harvested and post fixed overnight in the same fixative. The spinal cord segments were cut in serial 50µm thick parasagittal sections on a Vibratome (Leica Instruments, Germany), mounted onto gelatin-coated slides and coverslipped with DPX. The DRGs were cryoprotected in 20–30% sucrose for 2–3 days at 4C, embedded in Tissue-Tek (O.C.T., Miles Inc., Elkhart, IN, USA), 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 post-fixed for 2 h, cryoprotected in 10% and 20% sucrose for 48 hours and frozen in liquid isopentane. 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, post-fixed in 1% OsO4 in 0.1 M cacodylate buffer (pH=7.4), dehydrated in acetone, and embedded in Vestopal. Semi-thin 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 the results were expressed as left/right muscle weight ratios.

Morphological analysis after retrograde labelling - Paper II

Nuclear profiles of labelled sensory and motor neurons were counted in all sections at x250 final magnification in a microscope using filter block A (Leitz Aristoplan). The total number of nuclear profiles was not corrected for split nuclei, since there was uniformity in nuclear size and since 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 a physical dissector (Ma et al. 2001) and by counting neurons reconstructed from serial sections. Myelinated axons in the distal tibial nerve stamp were counted at x1000 final magnification using fractionator probe in Stereo Investigator™ 6 software (MicroBrightField, Inc.,USA).

Preparations were photographed with a Nikon DXM1200 digital camera attached to a microscope (Leitz Aristoplan). 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.

IN VITRO CHARACTERISATION OF CELLS

Characterisation of "stemness" - Paper V, VI

At passage 1, sub-confluent cultures were treated with agents to induce the phenotype of mesoderm-derived cells. For osteogenic induction, cultures were treated with 100 µg/ml ascorbate, 0.1 µM dexamethasone and 10 mM β-glycerophosphate for a period of 3 weeks. Cells were then fixed with 4% (w/v) paraformaldehyde for 30 minutes at room temperature, washed 3 times with phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin and then incubated with 1% (w/v) Alizarin Red solution to stain for calcium deposition. For induction of a chondrocyte phenotype, cells were treated with 0.1 µM dexamethasone, 50 µg/ml ascorbate, 10 ng/ml TGF-β1, 40 µg/ml proline and 1% (v/v) ITS™+ premix (BD Biosciences, UK) for 3 weeks. Cells were then fixed with 10% (v/v) formalin for 60 minutes, washed in H_2O and stained for proteoglycan with 1% (w/v) toluidine blue.

For immunocytochemical assessment of stem cell markers, cells cultured on slide flasks (Nunc-Fisher Scientific, UK) were processed essentially as for immunohistochemistry. Monoclonal stro-1 (1:50, R&D Systems, UK) or nestin (1:500, Chemicon, USA) antibodies were tested.

Immunocytochemistry and Western blotting - Paper V

Cells were incubated with antibodies for differentiation markers, mouse anti-glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA), rabbit anti-S100 (1:500; Dako, Denmark) and rabbit anti-p75 (1:500; Promega, USA) as for immunohistochemistry (see above). For western blotting, individual lysates were prepared from one 75 cm^2 flask of confluent cultures. Cells were washed in PBS and then scraped into buffer containing 100 mM PIPES, 5 mM MgCl, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100, 5 mM EGTA and protease inhibitors (Sigma, UK). Lysates were incubated for 15 minutes on ice and then subjected to 2 freeze-thaw cycles prior to analysis of protein content using a commercially available protein assay kit (Bio-Rad, UK). Fifteen µg protein was prepared per

sample, combined with Laemmli buffer and denatured at 95°C for 5 minutes. Proteins were resolved at 120 V on 15% (for S100) or 10% (for GFAP) sodium dodecyl sulphate-polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, membranes were blocked for 1 hour in 5% (w/v) non-fat dry milk in TBS-Tween (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween), and then incubated overnight at 4°C with either monoclonal anti-GFAP (1:200; LabVison, USA), monoclonal anti-S100 (1:750; Chemicon, UK) or polyclonal anti-p75 (1:250; Santa Cruz Biotechnology, USA) antibodies. Following 6 x 5 minute washes in TBS-Tween, membranes were incubated for 1 hour with HRP-conjugated secondary antibodies. Membranes were washed as previously and treated with ECL chemiluminescent substrate (Amersham, UK) for 1 minute and developed by exposure to Kodak X-OMAT light-sensitive film. Antibody was stripped from the membranes using 100 mM glycine pH2.9 and the blots re-probed with β-tubulin antibody (1:1000; Abcam, UK) as a loading control.

In vitro neurite outgrowth assay - Papers V and VI

ASC (rat omental derived or superficial and deep layer human derived) were plated at a density of 10000 cells per slide flask and allowed to settle for 24 hours. NG108-15 cells were then added to the ASC monolayer at a density of 1000 cells and the co-cultures maintained for a further 24 hours followed by fixation with 4% (w/v) paraformaldehyde for 20 minutes at room temperature. Fluorescent immunocytochemistry (as above) using mouse anti-neurofilament protein antibody (1:500; BioMol, UK) or monoclonal β-III tubulin (1:500; Sigma, UK) incubated at 4°C overnight followed by secondary labelling with goat anti-mouse Cy3 conjugate (1:200, Amersham Biosciences, UK) was used to visualise NG108-15 neurite outgrowth on the ASC. Cultures were examined using an Olympus IX51 inverted fluorescence microscope and images captured using Image ProPlus software (MediaCybernetics, Marlow, UK). The trace function was used to determine the percentage of NG108-15 cells expressing neurites and the number of neurites expressed per cell. For each experiment neurite data were ordered according to length (μm). The longest neurite in each experiment was thus identified and the mean of these calculated from 5

independent experiments. An average of one hundred NG108-15 cell bodies were analysed for each condition in each experiment.

Cell proliferation assay – Paper VI

The proliferation of human ASC isolated from both layers (superficial versus deep layer) was assessed over 96 hours using a CellTiter 96® Aqueous non-radioactive cell proliferation assay (Promega, USA). At passage 2, the cells were plated in a 96-well plate (200µl of medium containing 2,000 cells in each well and 200 µl of medium without cells for the control). At regular intervals (24h, 48h, 72h, 96h), 20 µl of Cell Titer 96® Aqueous Assay Reagents (Promega, USA) were added and incubated for 4 hours at 37°C and 5% CO_2. The absorbance was recorded at 490 nm using an ELISA plate reader. For the analysis, the average control absorbance was subtracted from the average cell absorbance.

RT-PCR – Paper VI

Total RNA was isolated from human ASC using an RNeasy™ kit (Qiagen, Sweden) and then 1ng RNA was incorporated into the One-Step RT-PCR kit (Qiagen) per reaction mix. Primers were manufactured by Sigma, UK (Table 1). A thermocycler (Biometra, Göttingen, 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), annealing (30sec, optimised per primer set as described in Table 2) 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, UK). Samples were visualised under UV illumination following GelRed™ nucleic acid stain (BioNuclear, Sweden) incorporation into the agarose.

Table 2. Primer sequences for RT-PCR and annealing temperatures used (˚C).

Factor	**Forward Primer (5'→3')**	**Reverse Primer (5'→3')**	**˚C**
oct4	GAAGCTGGAGAAGGAGAAGCTGG	CAAGGGCCGCAGCTTACACAT	63.0
nanog	CTCCTTCCATGGATCTGCTTATTC	GGTCTTCACCTGTTTGTAGCTGAG	61.0
NGF	ATACAGGCGGAACCACACTCAG	GTCCACAGTAATGTTGCGGGTC	64.0
BDNF	AGAGGCTTGACATCATTGGCTG	CAAAGGCACTTGACTACTGAGCATC	64.0
GDNF	CACCAGATAAACAAATGGCAGTGC	CGACAGGTCATCATCAAAGGCG	55.9
NT3	GGGAGATCAAAACGGGCAAC	ACAAGGCACACACACAGGAC	62.0
GAPDH	GAAGGTGAAGGTCGGAGT	CAAGCTTCCCGTTCTCAGC	62.0

Statistical analysis – All papers

One-way analysis of variance (ANOVA) parametric or non parametric (Kruskal-Wallis) tests with corresponding post hoc tests (Bonferonni, Newman–Keuls or Dunn Multiple Comparisons) (Prism®, Graph-Pad Software, Inc.; San Diego, California) were used where appropriate to determine statistical differences between experimental groups. Significance was determined as *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

RESULTS

NERVE CONDUITS FOR NERVE REPAIR (PAPERS I,II)

Comparisons were made between tubular PHB and fibrin conduits. The first study (Paper I) proved the concept and showed the potential of a conduit fabricated from fibrin glue (Tisseel®). It was possible to create a stable conduit that guided the nerve for the initial phase of nerve regeneration. The conduit supported axonal sprouting and did not collapse in the centre of the conduit.

Fibrin conduits showed after one week *in vitro* an intact structure with obvious porosity in the histological analysis. After 2 weeks *in vivo*, fibrin conduits were partially resorbed, with an approximate 20% reduction in the diameter of conduit. Conversely PHB conduits showed no signs of hydrolytic degradation. The use of single epineural sutures at surgery ensured there was no nerve detachment from the conduits and sustained stability of the junction nerve with the conduit. However, due to the adhesive capability of the fibrin conduit these sutures are not mandatory but they have been employed to equalise conditions at the ends of the conduits. There were also no signs of haematoma or infection in either type of conduits indicating there was a good tissue acceptance without altering the tissue environment around the implantation site.

After 2 weeks, nerves remained attached to both types of conduits and showed a growth cone directed towards the distal end. PGP9.5 immunohistochemistry indicated that the fibrin conduit showed significantly better axon regeneration distance than the PHB conduit (1.9mm vs. 4.1mm respectively, $P < 0.05$). S100 staining showed that axons which had not passed through the centre of the conduit did not cause the fibrin conduit to collapse. A distinct area could be identified where the conduit retains its lumen after the two weeks *in vivo*. This indicates that the fibrin conduit can provide sufficient rigidity *in vivo* to guide the regenerating nerve. Furthermore, the open lumen does not impede sprouting axons and allows the conduit to provide a permissive environment for regeneration. Histological analysis of the fibrin conduit revealed its porous

structure which would allow neurotropic growth factors to pass into the lumen of the conduit (Jubran and Widenfalk 2003). Conversely, the surface of the fibrin clot is cell repellent and prevents fibrous tissue to invade the lumen (Takazawa et al. 2005), and therefore the sprouting axons are provided with stimulating factors but not blocked by cell invasion. Also the Schwann cell regeneration was assessed proximally and distally using S100 staining. Analysis showed a superior cell intrusion within the fibrin conduit proximally (S100; 2.1mm vs. 4.2mm) and distally (S100; 1.7mm vs. 3.2mm).

In the long term evaluation in Paper II the proximal and distal nerve ends were shown to be connected by newly formed tissue containing a network of small blood vessels. Immunostaining of longitudinal sections prepared from the fibrin conduits and peripheral nerve grafts with antibodies against βIII-tubulin and S100 protein revealed numerous nerve fibres bridging the proximal and distal nerve stumps and associated with Schwann cells. In all the fibrin conduit and nerve graft preparations studied, regenerating fibres could be followed into the distal nerve. In control experiments, at 16 weeks after peripheral nerve grafting, 5184 (± 574 S.E.M.) sensory dorsal root ganglion neurons and 1001 (± 37 S.E.M.) spinal motor neurons had regenerated across the distal graft-nerve interface into the tibial nerve and were retrograde labelled with Fast Blue. The number of regenerating neurons was significantly lower in the fibrin conduit group when compared with the nerve graft control group. Implantation of the fibrin conduit promoted regeneration of 3096 (± 187 S.E.M.) sensory DRG neurons ($p < 0.05$; 60% of control) and 520 (± 85 S.E.M.) spinal motor neurons ($p<0.05$; 52% of control). This shows that the tubular fibrin conduit supported regeneration of about 40% of all spinal motor neurons projecting to the tibial nerve.

Specimens of the distal nerve were harvested 3-5mm from the graft-nerve interface and contained numerous myelinated nerve fibres. Although there were no marked differences in appearance and distribution of myelinated fibres between the experimental groups, quantitative analysis demonstrated that the

total number of myelinated fibres was lower in the fibrin conduit group when compared with the nerve graft control. Thus, after peripheral nerve grafting, the distal nerve contained 25,370 (± 686 S.E.M.) myelinated axons, whereas after fibrin conduit implantation the number of axons was reduced to 21,750 (± 1252 S.E.M.; 86% of control; $p<0.05$). However, when the proportion of myelinated axons was compared with the proportion of regenerating neurons the data revealed that axons in the distal nerve underwent highly significant axonal sprouting after both fibrin conduit and nerve graft transplantation.

Muscle weight of gastrocnemius and soleus muscles recovered to 59% of the contralateral side after peripheral nerve grafting. When these results were compared to muscle weight after fibrin conduit implantation a similar recovery was found for soleus muscle (89% of nerve graft control; $p>0.05$) whereas the gastrocnemius muscle weight reached 82% of the control group ($p<0.05$).

ADDITION OF CELLS TO CONDUITS (PAPERS III, IV)

In vitro fibrin matrix (Paper III)

Using a two step seeding procedure by coating PHB with thrombin and subsequent delivery of cells in fibrinogen, an optimal protocol was developed for producing constructs in accordance with a previously published study (Bensaid et al. 2003). Cells were retained within the grid created by the fibrin matrix thereby keeping them in the desired position. Importantly, cell viability was maintained as GFP-labeled MSC could clearly be observed after 72 h culture in the matrix. Furthermore when differentiated MSC (dMSC) were seeded in the fibrin matrix on PHB they retained their Schwann cell morphology and expressed S100 protein, a marker used to characterise the differentiation of MSC towards a SC phenotype.

Cell adherence and distribution on PHB conduits (Paper III)

Two experimental groups were analysed with MSC or SCs seeded into PHB conduits either using cell culture medium alone or in fibrin matrix. The adherence of the cells on the PHB was quantified using nuclear DAPI staining. Cells (undifferentiated MSC (uMSC), dMSC, SC) seeded onto PHB using fibrin showed significantly better adherence than those seeded in culture medium alone ($P < 0.05$). The cells adhered in the middle of the conduit to the fibrin grid and interlocked with the PHB on the side. Also conduits were injected *in vitro* with fibrin matrix containing cell suspensions in preparation for implantation *in vivo*. Firstly, the cell density within the conduit was analysed after 72 h *in vitro*. The central zone area inside the conduits had a cell coverage of 30.21% (±2.1) of the total PHB area in the group using fibrin matrix compared with a coverage of 21.72% (±1.8) in the group using growth medium ($P < 0.05$). Furthermore, the intensity analysis using polynomic mean value curve (Image Pro Plus®) showed that cells were located to the centre of the conduit.

In vivo nerve regeneration (Paper III)

At two weeks the cells were found to be attached on the PHB fibres and the fibrin matrix grid had almost completely dissolved. Immunostaining using PGP9.5 antibody was used to measure regeneration distance. Comparing the alignment of the axons, they appeared to be more organised in the SC group that in the dMSC group. The axonal regeneration distance was significantly greater ($p<0.05$) in the SC fibrin matrix group and in the dMSC fibrin matrix group when compared with the controls, empty PHB and PHB-fibrin matrix. The combination of fibrin matrix with cells had a significant regeneration effect in comparison with the conduits containing fibrin matrix alone, which showed a trend to better regeneration but no significant difference compared with the empty PHB conduit. Schwann cell localisation (S100 staining) at the proximal end of the conduit showed better penetration into the conduit for SC ($p<0.05$) and a strong trend for dMSC in fibrin versus empty conduits. Positive trends could also be demonstrated for the dMSC and SC fibrin matrix group for SC penetration at the distal stump. The SC penetration for the support of

regenerating axons in the conduit elicited by dMSC (proximal 3.30mm, distal 2.80mm) was comparable with that mediated by SC (proximal 3.40mm, distal 2.91mm) seeding.

Development of PHB strip model (Paper IV)

Strips of 14mm length and 2mm width of PHB were seeded with Schwann cells. After 72h *in vitro,* DAPI and S100 staining showed homogenous arrangement and good alignment of cells on PHB. On cross sections, integration of cells within the PHB fibres could be demonstrated. Native as well as DAPI staining analysis of the fibrin glue layer showed cell attaching to the PHB strip. The PHB strip was compared with PHB tubular conduits *in vivo.* At surgery, both types of nerve gap bridges were easy to handle and the fixation with the epineural suture was uneventful. Eleven animals survived and none displayed autotomy. One animal died from an unrelated cause post-operatively. At explantation, constructs were incorporated well with the nerve stumps and showed no signs of haematoma or infection. The strips were integrated with adjacent tissue. The PHB conduit was surrounded by adjacent tissue as the PHB strip, showing a nerve in continuity and being able to support axonal sprouting and provide a stable bridging of the nerve gap with no detachment of the construct.

All nerves stayed attached to the scaffolds and showed a growth cone. PGP9.5 immunohistochemistry showed a superior nerve regeneration on PHB strips compared with PHB conduit tubes. After 2 weeks axons had crossed the gap on the PHB strips whereas regeneration distance was 3.17 ± 0.32mm in the PHB conduits . Similarly, SC infiltration within the construct from proximal (S100; 3.40 ± 0.36mm vs. 10mm, crossed) and distal (S100; 2.91 ± 0.31mm vs. crossed) ends was increased on PHB strips. Qualitative analysis showed a wider area of regenerating axons in the conduits. The regeneration cone diameter was only 22% on PHB strips compared with the regeneration within the conduit.

NEW TYPES OF CELLS FOR NERVE REPAIR (PAPERS V,VI)

Characterisation of rat stem cell cultures (Paper V)

Rat visceral adipose tissue was enzymatically digested and then centrifuged to isolate the stromal cell fraction from mature adipocytes. After approximately 1 week in culture, cells from the stromal fraction formed confluent fibroblast-like monolayers on 75 cm^2 flasks. Cells were then trypsinised and plated for MTT proliferation assays. There was an apparent lag phase in growth of cells up to 48 hours after which time the rate of proliferation expanded more rapidly. Cells isolated from bone marrow exhibited a similar growth pattern, however, the overall proliferation rate of cells taken from adipose tissue was significantly faster in passage 1 cultures. Although the growth rate of passage 5 adipose cells in the lag phase was not different from bone derived cells they still proliferated significantly faster after 48 hours.

In order to determine whether the cells isolated from adipose tissue exhibited properties of mesenchymal stem cells, they were treated with agents known to induce differentiation to cells originating from the mesoderm. Osteogenic differentiation was confirmed by the production of calcium deposits detected with Alizarin Red and chondrocyte differentiation by the presence of toluidine blue-positive proteoglycans. Passage 1 cultures were examined for the presence of the stem cell marker, stro-1. A small proportion (11.38 ± 0.87%) of cells were positive for stro-1 in adipose cell cultures, a similar number to that found in bone marrow cultures. The number of cells expressing nestin, a putative marker of neural progenitors (Dahlstrand et al. 1995) was approximately three times more in cultures of cells taken from adipose tissue compared with those from bone (14.6 ± 0.8% vs. 5.1 ± 1.6% $P<0.01$).

Differentiation to a Schwann cell phenotype

ASC at passage 2 were treated with a mixture of glial growth factors for a period of two weeks after which time they were analysed morphologically and for the expression of the Schwann cell proteins, GFAP, S100 and p75. Cells cultured in the differentiation media changed from a fibroblast-like morphology to an

elongated spindle shape, similar to that of Schwann cells. Neither GFAP nor S100 protein was detected in undifferentiated cultures (uASC) but both were expressed in differentiated (dASC) cells. Quantitative analysis indicated that 81.5 ± 1.5% of the cells adopted a spindle-like morphology of which 44.5 ± 3.7% expressed GFAP. Almost all of these GFAP positive cells also stained for S100 protein (42.9 ± 3.3% positive). p75 expression was occasionally observed in uASC but was readily apparent in the cultures treated with glial growth factors (36.38 ± 3.3% positive). A small fraction (16.8 ± 1.4%) of treated ASC retained a fibroblast-like morphology, some of which expressed GFAP (5.9 ± 1.3%), S100 (3.4 ± 1.0%) and p75 (1.23 ± 0.38%) proteins. A minority of cells (1.7 ± 0.5%) displayed a rounded cell body with multiple processes. To confirm that the results obtained by immunocytochemistry were not due to an artefact of cellular shrinkage, western blotting was performed. Lysates of dASC but not uASC showed a GFAP-immunoreactive band corresponding to a molecular weight of 55 kDa. This was present in Schwann cell lysates together with an additional lower band which is likely to represent a proteolytic fragment or alternate transcript. S100 and p75 proteins were also detected in dASC but were absent in uASC.

Functional properties of differentiated cells

The ability of ASC to promote neurite outgrowth was determined by examining their interaction with NG108-15 cells, a motor neuron-like cell line (Jiang et al. 2003). uASC and dASC were plated on slide flasks to form monolayers and then NG108-15 cells were added. Computerised image analysis of co-cultures after 24 hours was used to quantify three separate parameters: percentage of cells extending neurites, number of neurites per cell and length of longest neurite. Comparisons were made with control cultures of NG108-15 cells grown alone and NG108-15 cells seeded with Schwann cells. A small fraction (22.0 ± 2.5%) of control NG108-15 cells extended neurites which was significantly increased to 69.1 ± 4.1% ($P<0.05$) and 57.6 ± 5.3% ($P<0.05$) in the presence of dASC and Schwann cells respectively. uASC had no significant effect. Likewise the number of neurites extended per cell was significantly ($P<0.05$)

increased in co-cultures of NG108-15 cells with dASC or Schwann cells, when compared with NG108-15 cells grown alone. The mean longest neurite extended by control cultures of NG108-15 cells was 67.5 ± 7.5 µm and in co-culture with uASC it was 74.3 ± 9.7 µm. In contrast, dASC evoked a significant ($P<0.01$) increase in neurite length to 205.2 ± 2.7 µm and Schwann cells stimulated an increase to 309.8 ± 31.5 µm ($P<0.01$).

Characterisation of human ASC cultures - Paper VI

1 g of adipose fat tissue biopsy was harvested from the two abdominal fat layers (superficial and deep) during the abdominoplasty procedure on 20 patients. After enzymatic digestion and centrifugation the stromal cell fraction was isolated from mature adipocytes and plated in a 75-cm^2 flask. After approximately 3 weeks in cultures, upon reaching confluence, cells were trypsinized and counted. At passage 1, the number of cells from the superficial layer was significantly ($p<0.05$) higher in comparison to the number of cells from the deep layer.

The proliferation rates of the ASC cells isolated from both layers (superficial versus deep layer) were compared at passage 2 during 96 hour using a CellTiter 96® Aqueous non-radioactive cell proliferation assay (Promega, USA). The cells from the superficial layers showed a trend of better proliferation after 96 hours in comparison to the deep layer and a significant difference for the proliferation between 24 and 96 hours in culture.

Cells isolated from the superficial and deep layer were both positive for stro-1 with a higher number of positive cells counted for the cells from the superficial layer (94.97% stro-1 positive cells in the superficial layer and 84.22% in the deep layer).

Neurotrophic activity of human ASC

To compare the ability of the cells from both layers (superficial vs. deep) to promote neurite outgrowth, the co-culture model of ASC and NG108-15 was utilized. The interaction between the cells isolated from the superficial layer and

the NG108-15 cells showed significantly ($p<0.05$) better results for neurite outgrowth in comparison with co-cultures using the cells from the deep layer. The number of NG108-15 cells expressing neurites increased in the presence of cells from the superficial layer (69.96%) in comparison with the co-cultures with the cells from the deep layer (13.22%). The average neurites length (for superficial: 77.59 µm and for deep: 45.3µm) and the average length of the longest neurites of the NG108-15 cells was significantly higher for the co-cultures with the cells from the superficial layer (for superficial: 348.76µm and for deep 134.07 µm).

The presence of various nerve growth factor gene transcripts was assessed using qualitative RT-PCR methodology. Transcripts for nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF) and neurotrophin-3 (NT3) were detected in both deep and superficial layers. RT-PCR amplification efficacy of the mRNA was confirmed by the amplification of the GAPDH housekeeping gene. Gene transcript levels were variable between patients and when analyzed semi-quantitatively (as a ratio of GAPDH levels) there was no significant difference in expression levels between the deep and superficial layers.

DISCUSSION

Empty artificial nerve conduits are sub-optimal because they do not replicate the cellular environment of an autologous nerve graft (the current gold standard treatment for gap injuries). Addition of SCs, the endogenous mediators of regeneration, can enhance their results in nerve gap repair. However, cultured SCs have limited clinical applications since the requirement for nerve donor material evokes some morbidity but the main problem is the need for an extended period of time to culture and expand the cells thus delaying treatment. Identification of novel cell-based therapies is therefore required to stimulate progress in developing tissue engineered nerve conduits. In this thesis it was shown that an optimized matrix and an optimized seeding of cells are beneficial for nerve regeneration using MSC in conduits.

MSC are an attractive cell source for the regeneration of nerve tissue as they are able to self-renew with a high growth rate and have multi-potent differentiation properties. Bone marrow has been used as a source of MSC which can be selectively differentiated into Schwann cells to enhance nerve regeneration in experimental animal models (Tohill et al. 2004). The studies in this thesis have shown MSC to be able to sustain morphology, adhere, differentiate and proliferate on the surface of fibrin glue *in vitro* and allow good cell integration *in vivo*. Fibrin was assessed as a tissue engineering material for a nerve gap conduit. The effect on short axon regeneration using a new fibrin glue tubular conduit was investigated. This novel technique was applied in an established rodent sciatic nerve gap model and compared with PHB, currently one of the most promising conduit material for nerve regeneration, and showed a significantly favourable result for the fibrin conduit. The long term evaluation of fibrin conduits showed significant benefit regarding axon regeneration. For humans the harvest of bone marrow MSC is an invasive and to some extent a painful procedure and alternative sources from which to isolate MSC should be investigated. Studies in this thesis showed that new cell types harvested from adipose tissue can potentially be used to enhance the application of the artificial nerve conduit.

Effects of a tubular fibrin glue conduit on axonal regeneration after peripheral nerve injury (Paper I,II)

It was hypothesised that a conduit constructed entirely from fibrin could be beneficial for nerve repair (Papers I and II). Recently, it has been shown using an experimental rabbit model that autologous fibrin glue is beneficial for peripheral nerve regeneration (Choi et al. 2005). SC suspended in 1 % fibrinogen, 2 % CaCl, 2 % gentamycin and 2 % of aprotinin can be added to the myelon of a rat and are able to proliferatec (Blits et al. 2004). In human cell studies, fibrin has been used successfully as a matrix for MSC where an optimal concentration of fibrinogen and thrombin has been evaluated to allow a good proliferation and fast resorption of the matrix (Bensaid et al. 2003).

The findings presented in Paper I suggest an additional significant advantage of the new fibrin conduit for the initial phase of peripheral nerve regeneration. The combined properties the fibrin conduit is not matched by any other previously described autologous material including collagen, carbonate, or alginate. This study thus shows that the use of fibrin glue as a conduit is a step towards a usable graft to bridge peripheral nerve lesions. This is even more interesting concerning the wide spread and acceptance of fibrin glue amongst the surgical community.

Effects of fibrin matrix supplemented with Schwann cells and bone marrow stem cells on peripheral nerve regeneration (Paper III)

Although previous studies have indicated a beneficial effect of transplanting cells suspended in cell culture medium, the hypothesis that loading nerve conduits with a matrix would enable a more homogenous distribution of cells and further promote regeneration was tested. SC or MSC were suspended in a fibrin matrix and injected into a PHB nerve conduit.

Initial *in vitro* observations showed that a two-step seeding procedure with an initial thrombin coating of the PHB and the later delivery of a cell-fibrinogen component is suitable for the seeding of cells in a nerve conduit. Cells adhere significantly better on PHB when mixed with a fibrin matrix. An optimal concentration of fibrinogen and thrombin that allows cell proliferation and fast resorption of the matrix was determined. The results were consistent with a

previous study indicating a 9/50 fibrin solution using Tisseel® fibrin glue is completely resorbed after 2 weeks and allows optimal cell proliferation (Labrador et al. 1998). Other groups have also shown the biocompatibility of fibrin with SC obtained from a variety of species including humans (Rodriguez et al. 2000). Other protocols have been applied to produce fibrin matrices. For instance, addition of thrombin (50 U/ml) to mixtures of fibrinogen (80mg/ml) plus fibrin stabilising factor XIIII (75 U/ml) and the plasmin inhibitor, aprotinin, produces a clotting time of 25 seconds. Under such conditions, MSC and β-tricalcium phosphates-fibrin suspensions can be used to produce bone matrix (Yamada et al. 2003).

The studies in Paper III use fibrin transplants with MSC differentiated towards a SC phenotype, a cell type which has been shown to have a beneficial effect on nerve regeneration (Tohill et al. 2004). These cells were compared with primary cultures of SC. Previous studies of dorsal root ganglion neurite outgrowth and myelination in fibrin matrices suggest that SCs have the capacity to migrate and make functional interactions with neurons (Corfas et al. 2004). Other i*n vitro* experiments have shown that alginate hydrogel, a naturally derived polysaccharide of non-animal origin, has the ability to host cultured Schwann cells (Mohanna et al. 2005). Matrigel has also been shown to enhance proliferation of SCs (Tohill et al. 2004) and MSCs (Mosahebi et al. 2001). *In vivo* experiments have demonstrated that Matrigel® alone (Labrador et al. 1998) and in combination with SCs (Rodriguez et al. 2000) or MSCs (Mimura et al. 2004) is able to support peripheral nerve regeneration (Qian and Saltzman 2004). Despite the obvious benefits of Matrigel, it is unlikely to be used in clinical applications since it is derived from a sarcoma cell line. Thus, encouraged by the initial *in vitro* observations regarding fibrin and cells and other studies showing that autologous fibrin glue is beneficial for peripheral nerve regeneration (Choi et al. 2005) PHB-fibrin matrix conduits were tested *in vivo.*

The first important finding was that PHB conduits loaded with fibrin matrix in the absence of cells did not inhibit regeneration. This is in contrast to previous studies using alginate gel (Mosahebi et al. 2003). Immunostaining using PGP9.5 antibody was used to measure regeneration distance. Comparing the alignment of the axons, they appeared to be more organised in the SC group that in the dMSC group. The axonal regeneration distance was significantly greater ($p<0.05$) in the SC fibrin matrix group and in the dMSC fibrin matrix group when compared with the controls empty PHB and PHB-fibrin matrix. The combination of fibrin matrix with cells has a significant regeneration effect in comparison with the conduits containing fibrin matrix alone, which shows a trend to better regeneration but no significant difference compared with the empty PHB conduit .Thus, dMSC were able to mimic the effects that SC have on axon elongation which could be an advantage in future clinical applications since MSC are easy to harvest and culture compared with SC.

Consistent with the enhanced axon regeneration (PGP9.5) there was an increased penetration of SC into the proximal stump (S100). Schwann cell localisation (S100 staining) at the proximal end of the conduit showed better penetration into the conduit for SC ($p<0.05$) and a strong trend for dMSC in fibrin versus empty conduits. Positive trends could be demonstrated for the dMSC and SC fibrin matrix group for SC penetration at the distal stump. The SC penetration for the support of regenerating axons in the conduit elicited by dMSC (proximal 3.30mm, distal 2.80mm) was comparable with that mediated by SC (proximal 3.40mm, distal 2.91mm) seeding. These effects are most likely due to axon/SC interactions occurring during the regeneration process (Hobson et al. 2000).

The use of artificial nerve conduits, such as PHB (Hazari et al. 1999; Atkins et al. 2006), is a new and promising approach for treatment of peripheral nerve injuries. Previously it was shown that addition of either SC or dMSC to PHB nerve conduits enhances nerve regeneration (Tohill et al. 2004). Compared with previous reports using cells delivered in growth medium alone (Mosahebi et al.

2001), greater axonal regeneration was induced by dMSC suspended in fibrin matrix. The conclusion from Paper III is that a fibrin matrix can be used to successfully seed regenerative cells within PHB nerve conduits. This is a further step towards the optimisation of artificial nerve conduits for the treatment of peripheral nerve injuries.

Comparison of PHB strip and PHB tubular conduit on peripheral nerve regeneration (Paper IV)

In Paper IV it was hypothesised that it might be possible to treat nerve injuries without the need for a tube like structure which is commonly used in nerve tissue engineering. It has been shown in a rodent experiment that peripheral nerve repair using a single-fascicle graft resulted in faster functional recovery and better morphometric outcome compared with conventional nerve grafting (Siemionow et al. 2004). The study suggests that a small structure is capable of guiding axons at a faster rate to their recipient muscle. Using this as a basis for the study in paper IV, the potential of an open, non-tube like structure seeded with SCs attached using fibrin glue (Tisseel®) was evaluated for the promotion of nerve regeneration.

The regenerating cone on the strip was smaller in diameter when compared with the conduit group. This might represent only a small number of regenerated axons which are unable to stimulate the muscle fully to contract but could preserve its properties at the neural endplate (Blits et al. 2004). These initial end organ connecting axons might lead a greater number of regenerating axons towards neural endplates.

A similar concept was introduced in 1995 by Whitworth et al. as fibronectin “mats” were successfully used to bridge 10 and 5 mm gaps, respectively, in rodent’s sciatic nerves (Whitworth et al. 1995). Recently, it has been shown, using a rabbit model, that autologous fibrin glue is beneficial for peripheral nerve regeneration (Choi et al. 2005). In other studies longitudinal sutures were tested using 7-15mm defects in rodents. Three loops of sutures were introduced using polyglactin and polyamide to enhance nerve regeneration. The gaps could be bridged with this technique but were significantly enhanced by placing a

segment of nerve graft as SC source in the middle of the defect (Scherman et al. 2000; Scherman et al. 2001). These studies support the concept of a SC strip as a guide for axon regeneration. The PHB strip fills the requirement of being a stable mechanical support for axonal growth whereas the seeded SCs produce the required neurotrophic factors stimulating the outgrowth of axons. In the present study fibrin glue seeded with SCs supported by a thin PHB strip achieved superior regeneration distance when compared with SC seeded PHB conduits. This newly described matrix strip seems to fit all the requirements for an optimal axonal sprouting and the achieved results suggest a significant advantage in the early phase of nerve regeneration. A possible factor leading to improved nerve regeneration by the use of a strip group might be an eased and superior vascularisation of this open construct (Hobson 2002).

Implantation of a conduit might be more traumatic than interposition of a strip because of a larger soft tissue mobilisation needed to fit the larger construct in. However in the long term the protective environment provided by a tube like structure might be favourable.

It has been shown that external factors and invasion of scar tissue, growth of the regenerating cone might be mechanically impeded (Hazari et al. 1999) and an insufficient number of axons might be able to reach the endplate not allowing recovery of function (Atkins et al. 2006). Therefore it is necessary to optimize the concentration and distribution of SC in the constructs as it is essential for successful nerve regeneration. This experiment proved that the use of optimally seeded SCs on a strip using fibrin glue matrix is superior compared with the SC seeded conduit.

Neuroregenerative potential of adipose stem cells (Paper V,VI)

It was previously shown that MSC derived from bone marrow can undergo differentiation to a Schwann cell phenotype (Dezawa et al. 2001). Given the clinical advantages of adipose tissue as an alternative source of stem cells (Gimble et al. 2007) the study described in Paper V was performed to determine whether it is also possible to derive Schwann cells from adipose tissue.

Rat visceral adipose tissue was enzymatically digested and then centrifuged to isolate the stromal cell fraction from mature adipocytes. In order to determine whether the cells isolated from adipose tissue exhibited properties of mesenchymal stem cells, they were treated with agents known to induce differentiation to various lineages. Cells also expressed the stem cell marker, stro-1 and nestin (a putative marker of neural progenitors). In contrast to the study by Ning et al., 2006 which identified all ASC as stro-1 positive, only a small fraction of cells in our cultures expressed this marker. This apparent discrepancy has also been reported by different groups examining human ASC (De Ugarte et al. 2003) and might reflect a difference in the region from which the tissue was obtained. There were approximately three times more nestin-positive cells in cultures of cells taken from adipose tissue compared with those from bone (14.6 ± 0.8% vs. 5.1 ± 1.6% $P<0.01$). Murine ASC have also been shown to express low levels of nestin which can be up-regulated upon neurogenic differentiation (Safford et al. 2002). These results suggest that ASC are not restricted towards specific mesodermal cell lineages and rather they retain some ability for differentiation along a neuro-glial lineage.

To investigate this further the ASC were exposed to a differentiation media previously used to induce Schwann cells from bone marrow MSC (Dezawa et al. 2001). Treated cells expressed glial proteins and exhibited morphological changes suggesting it is possible to produce high yields of Schwann–like cells from ASC. In contrast, previous reports have shown that neural induction media converts ASC to cells of a neuronal morphology with co-expression of GFAP, S100 and neuronal proteins including βIII-tubulin and neurofilament. Krampera recently showed these changes were rapid and reversible, suggesting a specific, full differentiation process (Krampera et al. 2007). To induce a more selective Schwann cell differentiation, the authors co-cultured various MSC with Schwann cells and this produced a long lasting expression of PMP-20 and S100 proteins in the absence of other CNS glial and neuronal markers. Interestingly, of all the MSC tested, those derived from adipose tissue produced the best

response. These effects could not be induced by Schwann cell factors alone, indicating contact between the two cell types was necessary. Whilst this suggests that some form of trans-differentiation might occur if ASC are transplanted at a nerve injury site *in vivo*, the methodology does not provide a suitable approach for the generation of clinically useful cells. Instead, in this study a defined mixture of GGF-2, bFGF and PDGF, molecules which are known to play a role in the differentiation and proliferation of Schwann cells was used (Jessen and Mirsky 1999). These molecules together with forskolin are responsible for the induction of the glial protein expression observed by immunocytochemistry. These results were confirmed by Western blotting; arguing against the notion suggested by Lu et al., 2004, that increased staining is merely the result of an increase in antigen levels per unit area, due to disruption of the cytoskeleton (Lu et al. 2004).

The function of the differentiated ASC was tested using a co-culture with the NG108-15 motor neuron-like cell line. The differentiated ASC evoked a similar response to Schwann cells, in that they promoted neurite outgrowth and elongation. NG108-15 cells have been shown to express low levels of trkA and GFR-α1, receptors for nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) respectively (Fu and Gordon 1997; Lee et al. 2006), two proteins which are known to be released by Schwann cells (Frostick et al. 1998). A recent study has shown that rat bone marrow cells constitutively express NGF, GDNF and brain derived neurotrophic factor (BDNF) (Yaghoobi and Mahani 2008). Human MSC also express NGF and BDNF although this is restricted to certain sub-populations . These and other unidentified factors promoted neurite outgrowth and survival in a co-culture system with SH-SY5Y cells (Crigler et al. 2006). Undifferentiated ASC had no effect on NG108-15 neurite outgrowth suggesting the process of differentiation led to the up-regulation of nerve stimulating factors.

These results suggest that ASC differentiated to a Schwann cell phenotype might have a beneficial role for treatment of peripheral nerve injuries. It was

previously shown that only differentiated bone marrow MSC, rather than untreated MSC, can stimulate nerve regeneration, a result in agreement with other groups. In contrast, other studies indicate that undifferentiated bone marrow MSC can also enhance regeneration and lead to improved motor function . This suggests that some form of trans-differentiation might occur in vivo as the result of local signals from injured Schwann cells and axons. The next goal is to understand the mechanisms of differentiation and how to generate clinically useful cells for the treatment of peripheral nerve injuries.

Human subcutaneous adipose tissue is an abundant and accessible source of multi-potent adult stem cells (Gimble et al. 2007). The adipose tissue is mainly obtained during liposuction or abdominoplasty and with the increased incidence of obesity in our society these operations are becoming more common, giving an increased availability for adipose tissue. In paper VI ASC were isolated from the two subcutaneous abdominal fat layers (deep and superficial) during abdominoplasty procedures and subsequently assessed the cells capacity to proliferate and their respective neurotrophic profiles. Cells cultured from the superficial layer were found to be a better source of ASC to use in nerve repair and other regenerative therapies.

The superficial layer of fat yielded the greatest number of cells and they proliferated significantly faster than ASC isolated from the deep layer. A recent study showed that there are significant differences in the concentration of cells which can be isolated from various body regions and that the lower abdominal region is the best source (Padoin et al. 2008). In contrast, another study showed no difference in viable cell number isolated from abdomen versus hip or mamma regions but did show that liposuction extracted cells proliferated at a slower rate than those obtained by resection (Oedayrajsingh-Varma et al. 2006). The ability of cells to differentiate into multiple lineages is determined by their expression of various “stemness” related transcription factors. Both deep and superficial layer cells expressed oct4 and nanog, consistent with previous studies of human ASC (Baglioni et al. 2009; Riekstina et al. 2009/9/22). Although the functional

role for these molecules in adult stem cells is controversial, studies in embryonic stem cells show that these factors act together to maintain pluripotency (Rodda et al. 2005/7/1). Furthermore, recent studies in ASC show that addition of exogenous oct4 improves cell proliferation and differentiation potency through epigenetic reprogramming of endogenous Oct4 and nanog genes (Kim et al. 2009). Having determined that cells isolated from the deep and superficial layers of fat showed a stem cell phenotype next their neurotrophic potential was investigated.

ASC isolated from the superficial layer of fat significantly enhanced neurite outgrowth compared with deep layer cells. The cells did not require any stimulation to elicit this response in contrast to studies with rat cells (Jiang et al. 2008; Xu et al. 2008) (Paper V). Untreated rat ASC can however protect against neuronal apoptosis, an effect in part mediated by their expression of soluble growth proteins such as insulin like growth factor (Wei et al. 2008). Analysis of neurotrophic factor RNA transcripts showed similar levels of NGF, BDNF, GDNF, and NT3 expression in both deep and superficial layer ASC. Although a factor which might account for the differences in neurite outgrowth of NG108-15 cells could not be identified, it is an important observation that human abdominal ASC express multiple growth factors which could be of benefit for neural repair. To date, most studies have focused on differentiating human ASC towards a neural phenotype for transplantation into injured patients (Safford et al. 2002; Anghileri et al. 2008; Franco Lambert et al. 2009). These studies have shown that treatment with a variety of induction protocols can lead to expression of neural and glial markers but they have not examined the neurotrophic potential of undifferentiated cells. Paper VI is the first to examine the neurotrophic factor profile of human ASC. Human ASC have recently been transplanted into athymic rats subjected to sciatic nerve transaction (Santiago et al. 2009). Treatment with the ASC enhanced nerve regeneration and reduced muscle atrophy but there was no evidence for an *in vivo* trans-differentiation of the cells towards a Schwann cell phenotype (Santiago et al. 2009). It is therefore likely that some of the benefits afforded by the cells were as a result of their

production of neurotrophic factors. Extracellular matrix molecules such as laminin and fibronectin can also potentiate nerve regeneration (Chernousov and Carey 2000; Chen et al. 2007). It is conceivable that differences between expression levels of these molecules in ASC isolated from the deep and superficial layers could account for the differences in neurite outgrowth in our *in vitro* model.

In conclusion the results of this study have shown that human ASC express a range of neurotrophic factors and cells isolated from the superficial abdominal fat layer promote neurite outgrowth *in vitro*. Together with their favorable proliferation kinetics, future *in vivo* nerve regeneration studies using human superficial layer ASC are warranted.

Clinical Implications

Despite improvements in surgical technique, surgeons still use autologous nerve grafts to bridge nerve defects, resulting in donor site morbidities and suboptimal functional recovery. Tissue engineering using a combination of nerve conduits and cell based therapies represents a new but as yet unproven approach to nerve repair. This project has shown that MSC could be of therapeutic use for the treatment of nerve injuries. Scientifically, the project defined novel lineage specific differentiation of MSC to a Schwann cell phenotype and these cells will have important clinical implications. Since nerve lesions are common within the population it is likely this research will have widespread health and socioeconomic consequences. Division of a peripheral nerve results in impaired sensation, reduced motor function and sometimes pain. Such injuries have a profound and permanent impact of the patient's life as they do not regain normal function. There are also serious economic implications for both the individual patient and society as a whole as a result of the intensive period of rehabilitation required. The results of this project define a new clinical possibility, using cells and novel conduits, to bridge nerve defects without implanting non human derived substances, allowing surgeons to address nerve gap lesions in a fast and

easy way. In summary, these experiments showed the extent of nerve regeneration in combination with cell biology and evaluated the effectiveness of using differentiated stem cells in conduits for nerve repair. This could allow new ways of treating nerve lesions providing a better outcome and minimal morbidity.

This thesis has focused on the regeneration that occurs at the site of the lesion after nerve injury. It has been shown that a new resorbable fibrin conduit enhances axon regeneration and that SCs and modified stem cells add the biological properties that are beneficial to the system. This implies a modern approach to heal nerves far from a mechanic adaptation but towards a biologically activated system that allows healing the site of injury and optimizing the regenerative potential of transected axons.

CONCLUSIONS

This thesis investigates the efficacy of fibrin conduits and matrix as well as the potential of rat and human ASC to regenerate nerves.

On the basis of the experimental data, the following conclusions can be made:

- The new fibrin conduit can be easily and stably sutured and is still present at 4 weeks but show signs of resorption. Compared with the PHB material the fibrin conduit shows enhanced axon regeneration both over a short term period (2 weeks) and also for a long term period (3 months).

- In the long term evaluation the fibrin conduit showed the number of distal myelinated axons was comparable to nerve autografts as was muscle weight recovery.

- Fibrin matrix attached cells to PHB enabling enhanced neural regeneration in the short term evaluation and it retains cells in the lumen of the conduit.

- A PHB strip seeded with regenerative cells provides a fast regeneration over a 10mm gap with quantitatively lower axons count. This can provide a fast connection to the end organs and might enable preservation of nerve conductive properties.

- ASC treated with glial growth factors differentiate to a Schwann cell phenotype characterized by expression of protein markers S100, GFAP, p75 and functional enhancement of neurite outgrowth in NG-108 co-cultures.

- The human superficial abdominal fat layer provides an ideal source of cells with neurotrophic properties which could benefit nerve repair.

ACKNOWLEDGEMENTS

During the years of research quite a few people have crossed my way and I would like to thank all members of the teams in Umeå, Lausanne and Manchester for contributing to this research.

Especially I would like to thank:

M. Wiberg, P.J. Kingham and G. Terenghi for being superb supervisors. Without all your help this work would not have been possible. Thank you for everything.

L. Novikov for correcting many of my manuscripts and great scientific support.

L. Novikova for teaching me the practical methods of retrograde labeling.

A. McGrath for being a great doctor and researcher that helped me significantly to complete the work.

J. Pettersson for being a great colleague that I meet in Manchester and Umeå and for our idea of the fibrin conduit.

G. Folkesson, M. Brohlin, G. Hällström, D. Wehlin, D. Mahay, C. Mantovani, A.L. Tallander for the steady support, work and scientific help.

G. Pierer, P.G. Di Summa, P. Erba, D. Schaakxs, H. Balcin, P. Engels, D.J. Schaefer, W. Raffoul, D. Egloff, D.A. McGrouther, R. Friis, for their constant support, suggestions and inspiration.

These studies were supported by the Swedish Medical Research Council, Umeå University, EU, County of Västerbotten, DAM, Baxter SA, Uni Basel, ICSF, Novartis Jubiläumsstiftung, Bangerter Stiftung, FAG, Swiss Life Fundation, Suva.

REFERENCES

Albala, D. M. and J. H. Lawson (2006). "Recent clinical and investigational applications of fibrin sealant in selected surgical specialties." J Am Coll Surg 202(4): 685-97.

Anghileri, E., S. Marconi, et al. (2008). "Neuronal differentiation potential of human adipose-derived mesenchymal stem cells." Stem Cells Dev 17(5): -.

Ansselin, A. D., T. Fink, et al. (1997). "Peripheral nerve regeneration through nerve guides seeded with adult Schwann cells." Neuropathol Appl Neurobiol 23(5): 387-98.

Atkins, S., K. G. Smith, et al. (2006). "The effect of antibodies to TGF-beta1 and TGF-beta2 at a site of sciatic nerve repair." J Peripher Nerv Syst 11(4): 286-93.

Atkins, S., K. G. Smith, et al. (2006). "Scarring impedes regeneration at sites of peripheral nerve repair." Neuroreport 17(12): 1245-9.

Baglioni, S., M. Francalanci, et al. (2009). "Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue." Faseb J: -.

Battiston, B., S. Geuna, et al. (2005). "Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair." Microsurgery 25(4): 258-67.

Be'eri, H., F. Reichert, et al. (1998). "The cytokine network of wallerian degeneration: IL-10 and GM-CSF." Eur J Neurosci 10(8): 2707-13.

Beazley, W. C., M. A. Milek, et al. (1984). "Results of nerve grafting in severe soft tissue injuries." Clin Orthop Relat Res(188): 208-12.

Bensaid, W., J. T. Triffitt, et al. (2003). "A biodegradable fibrin scaffold for mesenchymal stem cell transplantation." Biomaterials 24(14): 2497-502.

Bianco, P., M. Riminucci, et al. (1999). "Multipotential cells in the bone marrow stroma: regulation in the context of organ physiology." Crit Rev Eukaryot Gene Expr 9(2): 159-73.

Blits, B., T. P. Carlstedt, et al. (2004). "Rescue and sprouting of motoneurons following ventral root avulsion and reimplantation combined with intraspinal adeno-associated viral vector-mediated expression of glial cell line-derived neurotrophic factor or brain-derived neurotrophic factor." Exp Neurol 189(2): 303-16.

Brushart, T. M. (1993). "Motor axons preferentially reinnervate motor pathways." J Neurosci 13(6): 2730-8.

Bryan, D. J., K. K. Wang, et al. (1996). "Effect of Schwann cells in the enhancement of peripheral-nerve regeneration." J Reconstr Microsurg 12(7): 439-6.

Burnett, M. G. and E. L. Zager (2004). "Pathophysiology of peripheral nerve injury: a brief review." Neurosurg Focus 16(5): E1.

Caddick, J., P. J. Kingham, et al. (2006). "Phenotypic and functional characteristics of mesenchymal stem cells differentiated along a Schwann cell lineage." Glia 54(8): 840-9.

Chen, Z. L., W. M. Yu, et al. (2007). "Peripheral regeneration." Annu Rev Neurosci 30: -.

Chernousov, M. A. and D. J. Carey (2000). "Schwann cell extracellular matrix molecules and their receptors." Histol Histopathol 15(2): -.

Choi, B. H., S. G. Han, et al. (2005). "Autologous fibrin glue in peripheral nerve regeneration in vivo." Microsurgery 25(6): 495-9.

Corfas, G., M. O. Velardez, et al. (2004). "Mechanisms and roles of axon-Schwann cell interactions." J Neurosci 24(42): 9250-60.
Crigler, L., R. C. Robey, et al. (2006). "Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis." Exp Neurol 198(1): 54-64.
Dahlstrand, J., M. Lardelli, et al. (1995). "Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system." Brain Res.Dev.Brain Res. 84(1): 109-129.
De Ugarte, D. A., Z. Alfonso, et al. (2003). "Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow." Immunol Lett 89(2-3): 267-70.
Dellon, A. L. and M. E. Jabaley (1982). "Reeducation of sensation in the hand following nerve suture." Clin Orthop Relat Res(163): 75-9.
Dezawa, M., I. Takahashi, et al. (2001). "Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells." Eur J Neurosci 14(11): 1771-6.
Evans, P. J., R. Midha, et al. (1994). "The peripheral nerve allograft: a comprehensive review of regeneration and neuroimmunology." Prog Neurobiol 43(3): 187-233.
Fang, H., S. Peng, et al. (2004). "Biocompatibility studies on fibrin glue cultured with bone marrow mesenchymal stem cells in vitro." J Huazhong Univ Sci Technolog Med Sci 24(3): 272-4.
Franco Lambert, A. P., A. Fraga Zandonai, et al. (2009). "Differentiation of human adipose-derived adult stem cells into neuronal tissue: Does it work?" Differentiation 77(3): -.
Frostick, S. P., Q. Yin, et al. (1998). "Schwann cells, neurotrophic factors, and peripheral nerve regeneration." Microsurgery 18(7): 397-405.
Fu, S. Y. and T. Gordon (1997). "The cellular and molecular basis of peripheral nerve regeneration." Mol Neurobiol 14(1-2): 67-116.
Gibson, K. L., L. Remson, et al. (1991). "Comparison of nerve regeneration through different types of neural prostheses." Microsurgery 12(2): 80-5.
Gimble, J. M., A. J. Katz, et al. (2007). "Adipose-derived stem cells for regenerative medicine." Circ Res 100(9): -.
Gimble, J. M., A. J. Katz, et al. (2007). "Adipose-derived stem cells for regenerative medicine." Circ Res 100(9): 1249-60.
Guenard, V., N. Kleitman, et al. (1992). "Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration." J Neurosci 12(9): 3310-20.
Gupta, S. K., J. Pringle, et al. (1993). "Induction of myelin genes during peripheral nerve remyelination requires a continuous signal from the ingrowing axon." J Neurosci Res 34(1): 14-23.
Hall, S. (1997). "Axonal regeneration through acellular muscle grafts." J Anat 190 (Pt 1): 57-71.
Hazari, A., G. Johansson-Ruden, et al. (1999). "A new resorbable wrap-around implant as an alternative nerve repair technique." J Hand Surg [Br] 24(3): 291-5.
Hazari, A., M. Wiberg, et al. (1999). "A resorbable nerve conduit as an alternative to nerve autograft in nerve gap repair." Br J Plast Surg 52(8): 653-7.
Heumann, R., D. Lindholm, et al. (1987). "Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development,

degeneration, and regeneration: role of macrophages." Proc Natl Acad Sci U S A 84(23): 8735-9.
Hobson, M. I. (2002). "Increased vascularisation enhances axonal regeneration within an acellular nerve conduit." Ann R Coll Surg Engl 84(1): 47-53.
Hobson, M. I., C. J. Green, et al. (2000). "VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy." J Anat 197 Pt 4: 591-605.
Ide, C. (1996). "Peripheral nerve regeneration." Neurosci Res 25(2): 101-21.
Jessen, K. R. and R. Mirsky (1999). "Developmental regulation in the Schwann cell lineage." Adv Exp Med Biol 468: 3-12.
Jiang, J. X., R. C. Choi, et al. (2003). "Muscle induces neuronal expression of acetylcholinesterase in neuron-muscle co-culture: transcriptional regulation mediated by cAMP-dependent signaling." J.Biol.Chem. 278(46): 45435-45444.
Jiang, L., J. K. Zhu, et al. (2008). "Differentiation of rat adipose tissue-derived stem cells into Schwann-like cells in vitro." Neuroreport 19(10): 1015-9.
Jubran, M. and J. Widenfalk (2003). "Repair of peripheral nerve transections with fibrin sealant containing neurotrophic factors." Exp Neurol 181(2): 204-12.
Keskin, M., H. Akbas, et al. (2004). "Enhancement of nerve regeneration and orientation across a gap with a nerve graft within a vein conduit graft: a functional, stereological, and electrophysiological study." Plast Reconstr Surg 113(5): 1372-9.
Kim, J. H., M. K. Jee, et al. (2009). "Regulation of adipose tissue stromal cells behaviors by endogenic Oct4 expression control." PLoS.One. 4(9): e7166-.
Krampera, M., S. Marconi, et al. (2007). "Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus." Bone 40(2): 382-90.
Labrador, R. O., M. Buti, et al. (1998). "Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair." Exp Neurol 149(1): 243-52.
Le Guehennec, L., P. Layrolle, et al. (2004). "A review of bioceramics and fibrin sealant." Eur Cell Mater 8: 1-10; discussion 10-1.
Lee, R. H., W. L. Wong, et al. (2006). "Differential effects of glial cell line-derived neurotrophic factor and neurturin in RET/GFRalpha1-expressing cells." J Neurosci Res 83(1): 80-90.
Lindholm, D., R. Heumann, et al. (1987). "Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve." Nature 330(6149): 658-9.
Lu, P., A. Blesch, et al. (2004). "Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact?" J Neurosci Res 77(2): 174-91.
Lundborg, G. (1991). "Neurotropism, frozen muscle grafts and other conduits." J Hand Surg [Br] 16(5): 473-6.
Ma, J., L. N. Novikov, et al. (2001). "Delayed loss of spinal motoneurons after peripheral nerve injury in adult rats: a quantitative morphological study." Exp Brain Res 139(2): 216-23.
Mackinnon, S. E. and A. L. Dellon (1988). "A comparison of nerve regeneration across a sural nerve graft and a vascularized pseudosheath." J Hand Surg [Am] 13(6): 935-42.
McAllister, R. M. and J. S. Calder (1995). "Paradoxical clinical consequences of peripheral nerve injury: conduction of nerve impulses does not occur across the

site of injury immediately following nerve division and repair." Br J Plast Surg 48(6): 371-83.
Merle, M., A. L. Dellon, et al. (1989). "Complications from silicon-polymer intubulation of nerves." Microsurgery 10(2): 130-3.
Millesi, H. (2006). "Factors affecting the outcome of peripheral nerve surgery." Microsurgery 26(4): 295-302.
Mimura, T., M. Dezawa, et al. (2004). "Peripheral nerve regeneration by transplantation of bone marrow stromal cell-derived Schwann cells in adult rats." J Neurosurg 101(5): 806-12.
Mohanna, P. N., G. Terenghi, et al. (2005). "Composite PHB-GGF conduit for long nerve gap repair: a long-term evaluation." Scand J Plast Reconstr Surg Hand Surg 39(3): 129-37.
Mosahebi, A., M. Wiberg, et al. (2003). "Addition of fibronectin to alginate matrix improves peripheral nerve regeneration in tissue-engineered conduits." Tissue Eng 9(2): 209-18.
Mosahebi, A., B. Woodward, et al. (2001). "Retroviral labeling of Schwann cells: in vitro characterization and in vivo transplantation to improve peripheral nerve regeneration." Glia 34(1): 8-17.
Mueller, M., K. Wacker, et al. (2001). "Rapid response of identified resident endoneurial macrophages to nerve injury." Am J Pathol 159(6): 2187-97.
Oedayrajsingh-Varma, M. J., S. M. van Ham, et al. (2006). "Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure." Cytotherapy 8(2): -.
Ornelas, L., L. Padilla, et al. (2006). "Fibrin glue: an alternative technique for nerve coaptation--Part I. Wave amplitude, conduction velocity, and plantar-length factors." J Reconstr Microsurg 22(2): 119-22.
Padoin, A. V., J. Braga-Silva, et al. (2008). "Sources of processed lipoaspirate cells: influence of donor site on cell concentration." Plast Reconstr Surg 122(2): -.
Qian, L. and W. M. Saltzman (2004). "Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification." Biomaterials 25(7-8): 1331-7.
Riekstina, U., I. Cakstina, et al. (2009/9/22). "Embryonic Stem Cell Marker Expression Pattern in Human Mesenchymal Stem Cells Derived from Bone Marrow, Adipose Tissue, Heart and Dermis." Stem Cell Rev.Rep.: -.
Rodda, D. J., J. L. Chew, et al. (2005/7/1). "Transcriptional regulation of nanog by OCT4 and SOX2." J.Biol.Chem. 280(26): 24731-24737.
Rodriguez, F. J., E. Verdu, et al. (2000). "Nerve guides seeded with autologous schwann cells improve nerve regeneration." Exp Neurol 161(2): 571-84.
Rosberg, H. E., K. S. Carlsson, et al. (2005). "Prospective study of patients with injuries to the hand and forearm: costs, function, and general health." Scand J Plast Reconstr Surg Hand Surg 39(6): 360-9.
Rosberg, H. E., K. S. Carlsson, et al. (2005). "Injury to the human median and ulnar nerves in the forearm--analysis of costs for treatment and rehabilitation of 69 patients in southern Sweden." J Hand Surg Br 30(1): 35-9.
Safford, K. M., K. C. Hicok, et al. (2002). "Neurogenic differentiation of murine and human adipose-derived stromal cells." Biochem Biophys Res Commun 294(2): 371-9.

Santiago, L. Y., J. Clavijo-Alvarez, et al. (2009). "Delivery of adipose-derived precursor cells for peripheral nerve repair." Cell Transplant 18(2): -.
Scherman, P., G. Lundborg, et al. (2000). "Sutures alone are sufficient to support regeneration across a gap in the continuity of the sciatic nerve in rats." Scand J Plast Reconstr Surg Hand Surg 34(1): 1-8.
Scherman, P., G. Lundborg, et al. (2001). "Neural regeneration along longitudinal polyglactin sutures across short and extended defects in the rat sciatic nerve." J Neurosurg 95(2): 316-23.
Schlaepfer, W. W. and R. P. Bunge (1973). "Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture." J Cell Biol 59(2 Pt 1): 456-70.
Seddon, H. J., P. B. Medawar, et al. (1943). "Rate of regeneration of peripheral nerves in man." J Physiol 102(2): 191-215.
Shamash, S., F. Reichert, et al. (2002). "The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta." J Neurosci 22(8): 3052-60.
Siemionow, M., M. Zielinski, et al. (2004). "The single-fascicle method of nerve grafting." Ann Plast Surg 52(1): 72-9.
Strem, B. M., K. C. Hicok, et al. (2005). "Multipotential differentiation of adipose tissue-derived stem cells." Keio J Med 54(3): 132-41.
Sulaiman, O. A. and T. Gordon (2000). "Effects of short- and long-term Schwann cell denervation on peripheral nerve regeneration, myelination, and size." Glia 32(3): 234-46.
Sunderland, S. (1951). "A classification of peripheral nerve injuries producing loss of function." Brain 74(4): 491-516.
Sunderland, S. (1965). "The connective tissues of peripheral nerves." Brain 88(4): 841-54.
Sunderland, S. (1990). "The anatomy and physiology of nerve injury." Muscle Nerve 13(9): 771-84.
Takazawa, R., M. Yamato, et al. (2005). "Mesothelial cell sheets cultured on fibrin gel prevent adhesion formation in an intestinal hernia model." Tissue Eng 11(3-4): 618-25.
Tohill, M., C. Mantovani, et al. (2004). "Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration." Neurosci Lett 362(3): 200-3.
Tohill, M. and G. Terenghi (2004). "Stem-cell plasticity and therapy for injuries of the peripheral nervous system." Biotechnol Appl Biochem 40(Pt 1): 17-24.
Wei, X., Z. Du, et al. (2008). "IFATS Series: The Conditioned Media of Adipose Stromal Cells Protect Against Hypoxia-Ischemia-Induced Brain Damage in Neonatal Rats." Stem Cells: -.
Whitworth, I. H., R. A. Brown, et al. (1995). "Orientated mats of fibronectin as a conduit material for use in peripheral nerve repair." J Hand Surg [Br] 20(4): 429-36.
Xu, Y., Z. Liu, et al. (2008). "Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells in vitro." BMC Neurosci 9: 21.
Yaghoobi, M. M. and M. T. Mahani (2008). "NGF and BDNF expression drop off in neurally differentiated bone marrow stromal stem cells." Brain Res 1203: 26-31.

Yamada, Y., J. S. Boo, et al. (2003). "Bone regeneration following injection of mesenchymal stem cells and fibrin glue with a biodegradable scaffold." J Craniomaxillofac Surg 31(1): 27-33.
Young, V. L., R. C. Wray, et al. (1980). "The results of nerve grafting in the wrist and hand." Ann Plast Surg 5(3): 212-5.