The use of adipose derived stem cells in spinal cord and peripheral nerve repair

Mallappa K. Kolar

Department of Integrative Medical Biology
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Umeå University, Umeå 2014

LICENTIATE DISSERTATION IN MEDICAL SCIENCE

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ISBN: 978-91-7601-007-5
Printed by: Print and Media, Umeå, Sweden 2014

"I am thankful to all those who said no to me,
It's because of them, I did it myself"

-Albert Einstein

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ABSTRACT

Clinically, injuries affecting the spinal cord or peripheral nerves can leave those affected with severe disability and, at present, there are limited options for treatment. Peripheral nerve injury with a significant gap between the proximal and distal stumps is currently treated with autologous nerve grafting but this is limited by availability of donor nerve and has associated morbidities. In contrast, injuries to the spinal cord lead to an inhibitory environment caused by the glial cells and thereby, limit potential axonal regeneration. This thesis investigates the effects of human adipose derived stem cells (ASC) on regeneration after peripheral nerve and spinal cord injury in adult rats.

Human ASC expressed various neurotrophic molecules and growth factor stimulation of the cells *in vitro* resulted in increased secretion of BDNF, GDNF, VEGF-A and angiopoietin-1 proteins. Stimulated ASC also showed an enhanced ability to induce capillary-like tube formation in an *in vitro* angiogenesis assay. In contrast to Schwann cells, ASC did not induce activation of astrocytes and supported neurite outgrowth from the adult rat sensory DRG neurons in culture.

In a peripheral nerve injury model, ASC were seeded into a fibrin conduit, which was used to bridge a 10mm rat sciatic nerve gap. After 2 weeks, ASC enhanced GAP-43 and ATF-3 expression in the spinal cord, reduced c-jun expression in the DRG and increased the vascularity of the fibrin nerve conduits. The animals treated with stimulated ASC showed an enhanced axon regeneration and reduced caspase-3 expression in the DRG.

After transplantation into the injured C3-C4 cervical spinal cord, ASC continued to express neurotrophic factors and laminin and stimulated extensive ingrowths of 5HT-positive raphaespinal axons into the trauma zone. In addition, ASC induced sprouting of raphaespinal terminals in C2 contralateral ventral horn and C6 ventral horn on both sides. Transplanted cells also changed the structure and the density of the astroglial scar. Although the transplanted cells had no effect on the density of capillaries around the lesion site, the reactivity of OX42-positive microglial cells was markedly reduced.

Keywords: Spinal cord injury, peripheral nerve injury, adipose derived stem cells, regeneration, neurotrophic factor, angiogenic factor

ORIGINAL PAPERS

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

I. Kingham P.J., **Kolar M.K.**, Novikova L.N., Novikov L.N. and Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose derived stem cells enhances nerve repair. *Stem Cells and Development*, Nov 22, [Epub ahead of print], doi:10.1089/scd.2013.0396, 2013.

II. **Kolar M.K.**, Kingham P.J., Novikova L.N., Wiberg M. and Novikov L.N. The therapeutic effects of human adipose derived stem cells in a rat cervical spinal cord injury model, 2014, Manuscript resubmitted to *Stem Cells and Development* following revision.

ABBREVIATIONS

ASC	Adipose derived stem cell
BDNF	Brain-derived neurotrophic factor
BMSC	Bone marrow mesenchymal stem cell
CNTF	Ciliary neurotrophic factor
CsA	Cyclosporine A
CSPG	Chondroitin sulphate proteoglycans
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal root ganglion
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra acetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether)-tetraacetic acid
EGF	Epidermal growth factor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
GDNF	Glial cell derived neurotrophic factor
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
LIF	Leukaemia inhibitory factor
MSC	Mesenchymal stem cells
NGF	Nerve growth factor
NT-3	Neurotrophin-3
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PDL	Poly-D-lysine
PDGF	Platelet derived growth factor
PLL	Poly-l-lysine
RECA-1	Rat endothelial cell antigen-1
SC	Schwann cell
VEGF	Vascular endothelial growth factor

INTRODUCTON

1. Clinical background and epidemiology

Traumatic injuries to the ***spinal cord*** (SCI) affects several thousand people worldwide every year with an incidence of between 8 and 246 per million population globally, depending on the economic situation of the country [1]. The vast majority occur at the cervical level (C1-C7/T1; 55-65%), which is associated with a mortality rate of 10-15% within the first year. Long term, the expected lifespan of such patients is 10-15 years. There are two peaks for age of incidence of SCI; between 16-29 and >65 [2], with the primary cause of injury being motor vehicle collisions and falls, respectively [3]. The sex ratio varies between 2-6.7:1 (Male:Female; M:F) [2]. As a result of SCI, the patient can be left with profound disability. In view of current advances in medical care, technology and rehabilitation, the lifetime costs for each patient is estimated to be $25 million, amounting to $10 billion spent per year in the USA alone on such cases [4].

Peripheral nerve injuries (PNI) occur more frequently, with an annual incidence of around 13.9 per 100,000 [5]. Again such injuries predominantly affect young males (20-24). The majority of injuries affect the upper limb (70% of total) particularly the hand & wrist (63% of total). The vast majority (70%) of those with PNI are of working age (20-64) [5]. In digital nerve injuries, Thorsen et al found that the direct costs of digital nerve injuries, including hospital stay, operation, outpatient visits, visits to a nurse and/or a hand therapist amounted to €2653 EUR (range: €468-6949). The majority of patients (79%) lost time from work (median 59 days; range: 3-337) and some suffered from permanent nerve dysfunction despite surgical repair. These included problems with activities of daily living and subjective complaints of **numbness**, cold sensitivity and pain [6]. Although hospitalisation and immediate costs may be lower than SCI, follow-up and rehabilitation times can impact negatively on the patients ability to earn an income and further drain resources.

2. Pathophysiology of spinal cord and peripheral nerve injury

2.1. Spinal cord injury

The effects of SCI occurs as a result of two phases. Primary damage occurs as a result of direct trauma (sharp injury/compression/contusion) [7]. The mechanical injury leads to secondary injury involving biological sequences, beginning minutes after the primary injury continue up to years later. Although the processes are not distinct, secondary damage is divided into three phases; acute, sub-acute and chronic.

2.1.1. Acute and sub-acute spinal cord injury

Vascular effects: This includes haemorrhage, vasospasm, thrombosis, and loss of autoregulation, breakdown of blood - brain barrier and infiltration of inflammatory cells. The result is oedema, necrosis and ischaemia [8-10].

Free radical formation and lipid peroxidation: Production of free radicals cause oxidative death in spinal cord neurons and reduce the spinal cord blood flow leading again to oedema and an inflammatory response [11-13]. Free radicals react with the polyunsaturated fatty acid of the cellular membrane leading to peroxidation and disruption of the normal phospholipid architecture of cellular and subcellular organelle membranes. Lipid peroxidation then induces the formation of aldehyde products that impair the function of key metabolic enzymes, such as $(Na^+ + K^+)$-ATPase [14]. This enzyme activity is critical for the maintenance of neuronal excitability, and its failure results in loss of neuronal function and may ultimately lead to tissue dissolution [15].

Disruption of K^+, Na^+, Ca^{2+} ionic balance: This can affect depolarisation of cell membranes, ATPase failure and increase of intracellular Ca^{2+} [16] and thus, potentiate cell death.

Glutamate excitotoxicity: Following SCI there is an increased release of extracellular glutamate that induces excessive activation of glutamate receptors leading to further neuronal cell death [17, 18].

Apoptosis: There is strong morphological and biochemical evidence for the occurrence of apoptosis in neurons, oligodendrocytes, microglia and possibly astrocytes after SCI [19]. The death of oligodendrocytes in white matter tracts continues for many weeks after injury and may contribute to post-injury demyelination. Apoptotic cells were found after SCI in rodents, monkeys and humans, indicating that active cell death facilitates damage after CNS injury [20, 21]. The mediators of apoptosis after SCI are not well understood, but there is a close relationship between microglia and dying oligodendrocytes, suggesting the involvement of microglial cell activation [22].

Inflammatory response: Following trauma, rapidly infiltrating blood-derived macrophages and resident microglia become activated and release an increased amount of cytokines and reactive oxygen species [23]. This promotes further extravasation of leukocytes and increases damage to the tissue [24]. Conversely, inflammation can also play an important role in neural tissue repair by offering neuroprotection and/or neurotrophic support [25]. The timing of the inflammatory response may be a critical variable in determining if an inflammatory response has negative or beneficial consequences.

2.1.2. Chronic spinal cord injury

During the chronic phase, white matter demyelination, grey matter dissolution, connective tissue deposition and reactive gliosis occurs which leads to fibrotic and glial scar formation. The scar acts like a physical barrier, preventing axons to grow through it. It is formed predominantly by meningeal fibroblasts, blood-derived macrophages, reactive astrocytes and microglia. Reactive astrocytes produce growth-inhibitory extracellular matrix molecules including chondroitin sulphate proteoglycans [26]. In approximately 25% of patients, the glial scar surrounds a cystic cavity that progressively expands leading to syringomyelia. Finally, SCI can lead to the development of neuropathic pain and depression [27].

2.2. Peripheral nerve injury

Classification of peripheral nerve injury is based on the work of Seddon and Sunderland [28, 29] describing how different extent of damage to the nerve structures correlates with increased severity and thereby decreasing prognosis for functional recovery. Similar to SCI, damage to peripheral nerves can occur as a result of compression, contusion or transection. Injuries can range from compression of the nerve with no loss of continuity (neurapraxia) to complete transection of the nerve with no continuity of any structure (epineurium, perineurium, endoneurium, axons; neurotmesis) and affects both primary sensory and motor neuronal cell bodies reside in dorsal root ganglion (DRG) and spinal cord, respectively.

In the most severe form of injury (transection), Wallerian degeneration occurs, which involves physical fragmentation of axons and myelin distal to the site of injury within hours of injury. By 48-96 h post-injury, axonal continuity is lost and conduction of impulses is no longer possible. Myelin disintegration lags slightly behind that of axons but is well underway by 36-48 h. Schwann cells (SC), which normally surround the axons, play a key role in Wallerian degeneration. They initially become active within 24 h of injury. They divide rapidly to form dedifferentiated cells that upregulate expression for a range of molecules to assist in the degeneration and repair process. At an early stage, the role of the Schwann cell is to help remove the distal axonal and myelin debris which is then further processed by macrophages. At later stages, Schwann cells align themselves to form bands of Büngner. By this time, the macrophages have migrated into the injury zone, primarily through a haemopoietic route, passing through the walls of capillaries, which have become permeable locally. Thus, Schwann cells and macrophages work together to phagocytose and clear the site of injury in a process that requires 1 week to several months [30]. Endoneurial mast cells also play a pivotal role during Wallerian degeneration, proliferating dramatically within the first 2 weeks post injury. They release histamine and serotonin, which enhance capillary permeability and facilitate macrophage migration. During the initial stages, the endoneurial tubes swell in response to the trauma, but after the first 2

weeks they become smaller in diameter. By 5-8 weeks, the degenerative process is usually complete, and only remnants, composed of Schwann cells within an endoneurial sheath, remain.

Intrafascicular injuries involve retraction of the severed nerve fibre ends due to the elastic endoneurium. Local vascular trauma leads to haemorrhage and oedema, which result in a vigorous inflammatory response. Fibroblasts proliferate, and a dense fibrotic scar causes a fusiform swelling of the injured segment. Interfascicular scar tissue also develops so that the entire nerve trunk, which is left in continuity, is permanently enlarged. Often, it is adherent to perineural scar tissue as well [30].

3. Regeneration following spinal cord and peripheral nerve injury

3.1. Regeneration after spinal cord injury

Recovery following SCI has been reported to primarily occur due to plasticity in supraspinal and spinal networks. This was first suggested by the observation that paralysed cats with complete spinal cord transection could be trained to walk on a treadmill [31] demonstrating the fact that plasticity is significantly influenced by physical activity. The finding that the spinal cord had plastic properties led to novel rehabilitation strategies for humans with SCI. Patients with incomplete SCI achieved significant functional benefits by daily training on a moving treadmill [32] with locomotion training becoming routine for incomplete SCI patients all over the world.

Studies in animal models demonstrated that the spinal cord plasticity may occur through sprouting of spared axonal tracts that cross from the contralateral to the injury side [33], of spinal interneurons [34], adaptations of the motor neurons caudal to the injury [35], and by functional redundancy [36]. More recently, axonal regeneration in SCI has been shown to be possible, when the inhibitory environment of the glial scar is modified [37].

3.2. Regeneration after peripheral nerve injury

In complete transection, surgical re-anastomosis is the only method of treatment. In cases where a gap greater than 5mm exists between the two ends of the nerve bridging strategies have to be considered [38]. The current gold standard treatment in such cases is to use a nerve graft to anastomose the proximal and distal ends of the injured nerve. There are a number of drawbacks to this including limited availability of autologous nerves and associated morbidities including neuromas, loss of sensation and donor site tenderness. In addition, the regeneration is unlikely to yield a return to normal function due to mismatches [39].

Since the process of regeneration following transection is only able to take place once Wallerian degeneration is completed, the entire process of repair may last upto months after the injury, depending on the distance between the injury zone and target organ. The sequence of regeneration can be divided into anatomical zones; the neuronal cell body; the area between the cell body and the injury zone; the injury zone itself; the area distal to the injury zone upto the end organ and finally the end organ itself [30]. Changes occur to allow for axonal regeneration beginning from the neuronal body, with multiple axons sprout from each endoneurial sheath until they make contact with aligned Schwann cells in the bands of Büngner. Axonal regeneration occurs until a target organ is reached. However, regeneration is not always well directed as sensory axons may reach motor targets and vice versa. This can limit the clinical outcomes despite surgery.

Ultimately, successful regeneration is dependent upon axons of motor, sensory and autonomic type making appropriate connections first with the distal nerve stump and, finally, with their target organs. An understanding of the processes occurring during regeneration allows us to better target research towards possible supportive and/or replacement measures.

3.3. Biosynthetic conduits for peripheral nerve repair

In order to overcome the limitations of nerve autografting, alternative nerve conduit structures have been developed. Most of these involve the simple concept of using a tubular structure to connect the transected nerve ends. Regeneration in the hollow tube differs from primary nerve repairs in a number of ways due to the dead space between the two ends of the nerve. This process can be divided into five phases as follows: (i) fluid phase; (ii) matrix phase; (iii) cellular migration phase; (iv) axonal phase; and (v) myelination phase [38].

In the fluid phase, the influx of exudates from the proximal and distal stumps into the conduit occurs. This is rich in neurotrophic factors and extracellular matrix (ECM) precursor molecules, where peak concentrations are between 3-6 hrs. Following this, acellular bands of fibrin, running between the proximal and distal stumps, form from the converted ECM precursor molecules over the next week. Over the second week, this ECM framework provides a pathway for the Schwann cells, fibroblasts and endothelial cells to migrate along. This provides the guidance for the axonal growth toward the distal stump, a process that occurs over the next 2-4 weeks. By this time the fibrin cable has been degraded due to completion of cellular migration. Once the axonal phase is complete, the SCs switch from a proliferative to myelinating phenotype, resulting in some functional recovery over 6-16 weeks post repair. This process is limited to injury gaps of 4 cm in humans and 2 cm in rats in hollow tubular conduits. Beyond this distance of injury, there is limited to no regeneration [40].

A major development in the construction of biosynthetic nerve guidance conduits has been the progression from the simple concept of tubulisation to the creation of a conduit that more closely mimics the nerve environment. The ideal conduit should be biocompatible, biodegradable, permeable and exhibit certain biomechanical and surface properties such as flexibility, limited swelling, a predictable degradation rate and if possible, be transparent [41, 42]. This has meant a threefold approach to construction of a conduit through optimisation of the conduit's body, the intraluminal structure and the intraluminal contents (e.g. by addition of growth factors or regenerative cells).

4. Cell transplantation to promote spinal cord and peripheral nerve repair

To improve clinical outcomes, it will be necessary to enhance both axonal regeneration and neuronal survival, modulate organisation of the central nervous system, and inhibit or reduce atrophy of the target organ [43, 44]. One method of targeting these different needs may be through transplantation of stem cells [45]. The ideal stem cell in these cases would have certain characteristics, namely; be easily accessible, grow rapidly and in a controlled manner *in vitro*, be capable of modulating the immune reaction, and when transplanted *in vivo*, survive and contribute to new tissue formation [46]. Both embryonic and adult tissue-derived stem cells have been shown to promote nervous system regeneration [46], but widespread use of embryonic stem cells is unlikely given the ethical issues concerning their derivation. Adipose-derived stem cells (ASC) have multi-lineage (mesodermal and possibly ectodermal and endodermal) differentiation potential [47] and show a number of distinct advantages compared with other adult stem cell types. Adipose tissue is easily accessible via liposuction or abdominoplasty and has been shown to contain a higher number of stem cells/progenitors than bone marrow tissue [48]. The extracted cells also expand more rapidly than those from other tissues [49]. Encouragingly, recent studies have demonstrated the ability of ASC to support axonal regeneration after peripheral nerve injury[46, 50, 51], and this effect has been primarily attributed to the environmental support provided by the ASC during regeneration. ASC have also demonstrated the ability to promote angiogenesis, neurotrophic factor provision, and protection of neurons and improve the ability to protect target organs [52, 53].

AIMS OF THE STUDY

The specific aims of the study were:

- To evaluate the neurotrophic and angiogenic properties of human ASC and their effects on the retrograde reaction in sensory and motor neurons and axonal regeneration in a peripheral nerve injury model in adult rats (Paper I).

- To investigate the effects of human ASC on axonal regeneration, glial cell reactions and functional recovery after transplantation into the injured rat spinal cord (Paper II).

MATERIALS AND METHODS

1. Cell culture

1.1. Culture of human adipose derived stem cells

Human adipose tissue samples were obtained from abdominal fat from a total of patients undergoing elective surgery. Procedures were approved by the Local Ethical Committee for Clinical Research in Umeå University (No. 03-425). Adipose tissue was minced, digested with 0.15% (w/v) type I collagenase and centrifuged as previously described [54]. The final stromal fraction pellet containing stem cells was resuspended in growth medium (alpha Minimum Essential Medium; α-MEM, 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (Invitrogen Life Technologies, Sweden) and plated on a 75cm^2 tissue culture flask. Each cell pellet was obtained from approximately 2g of starting adipose tissue. The flasks were washed with Hank's balanced salt solution every 24 hours for three days to eliminate haematopoietic cells, after which fresh growth medium was added. Upon reaching confluence, cells were detached from the flasks using trypsin/EDTA and replated at a density of 5000cells/cm^2. Cells were maintained at 37°C and 5% CO2 and at passage P2-P6 were used for *in vitro* and *in vivo* experiments.

For Paper I, ASC were treated to stimulate an enhanced neurotrophic and angiogenic phenotype, as previously described for rat cells [55]. The growth medium was replaced with fresh medium containing 1mM β-mercaptoethanol and cultured for 24 h. Fresh medium containing 35 ng/mL all-trans-retinoic acid was replaced for 72 h. Subsequently, the cells were washed with HBSS and the medium was replaced by stimulation medium that consisted of growth medium with 200 ng/mL neuregulin1-β1 (R&D Systems), 10 ng/mL basic fibroblast growth factor (FGF-2; Milipore), 5 ng/mL platelet-derived growth factor (PDGF-AA; Millipore), and 14 mM forskolin (Sigma). Fresh stimulation medium was added every 72 h over a period of 2 weeks, and cells were passaged once they became 90% confluent. Cultures of unstimulated cells were always run in parallel. Experiments studying conditioned media (CM) effects were started after this first 2 week period of expansion, and cells were freshly plated and stimulated with factors. Cells for *in vivo* transplantation in paper I were also obtained after the 2 weeks' growth period and freshly stimulated for 48 h before transplantation. To define the isolated ASC as a non-haematopoietic and stromal/stem cell population, cells were immunostained with a Mesenchymal Stem Cells Characterisation Kit (Millipore AB, Sweden). The multipotency of the ASC was demonstrated by differentiation into osteogenic, and adipogenic lineages as described previously [55].

1.2. Culture of dorsal root ganglion neurons and neurite outgrowth assay

Cultures of dorsal root ganglion (DRG) neurons were prepared from adult rats (n=6) as described previously [56, 57]. The DRG neurons were cultured in Neurobasal^{TM}-A medium with B-27 supplement (Invitrogen Life Technologies, Sweden) and 0.5mM L-glutamine (SigmaAldrich, Sweden AB). For the neurite outgrowth assays in Paper I, the DRG neurons were plated on sterile 12mm circular coverslips in triplicate that were placed in a 35mm Petri dish. ASC at a density of 1.25 x 10^5cells/mL were plated on six-well plates in control or stimulation medium. Conditioned media (CM) from these wells were collected at 48 h, centrifuged at 650 g for 10min to remove any floating cells, and then, 1mL of the supernatant were applied directly to the Petri dishes containing DRGs, which had been plated 24 h earlier. CM and DRG co-cultures were maintained in the incubator for 24 h before the DRGs were fixed with 4% paraformaldehyde and immunostained with β-III-tubulin antibody. The resulting slides were observed with an ECLIPSE 90i microscope, and images were captured with Nikon Elements Imaging software (Nikon). The neurite outgrowths of β-III-tubulin-positive DRG were manually traced with Image Pro-Plus software (MediaCybernetics). The mean longest neurite length and total neurite outgrowth were determined. The experiments were repeated using ASC from different patients.

1.3. Culture of cortical astrocytes

For Paper II, primary cultures of astrocytes were prepared from cerebral cortices obtained from 5-day-old rats as described previously [58]. Cells from 2 rats were resuspended in growth medium (Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin) and plated onto a 75cm^2 poly-l-lysine-coated (PLL) tissue culture flask. When cells reached confluence after 8 days, the flasks were shaken at 150rpm for 4 hours to detach the microglia. The cells were cultured for an additional 14 days. The resulting culture contained 95% GFAP-positive astrocytes and 5% OX42-positive microglial cells.

1.4. Culture of Schwann cells

For Paper II, primary cultures of Schwann cells were prepared from the sciatic nerves of adult rats as described previously [59]. In brief, sciatic nerves were dissected into DMEM with 10% (v/v) FCS, cut into small pieces and incubated at 37ºC and 5% CO2 for 2 weeks. After enzyme digestion and trituration, the cells were plated onto 25cm² poly-D-lysine (PDL) coated tissue culture flasks with Schwann cell growth medium, supplemented with 10μM forskolin (SigmaAldrich) and neuregulin NRG1 (R&D Systems, UK). The purity of Schwann cells was assessed using immunostaining for low affinity NGF p75 receptors and was approximately 95%. Schwann cells at passage 3 were used for the *in vitro* experiments.

1.5. Confrontation assay of astrocytes with ASC and Schwann cells

For Paper II, confrontation assays were carried out by dropping a 50µl astrocyte cell suspension (~10^5 cells) at one end of a PLL-coated coverslip, and a glass strip used to smear the drop toward the centre to generate a straight edge. A second 50µl drop containing the same amount of either ASC or Schwann cells was placed at the opposite end of the coverslip and smeared to give a parallel straight edge, between 0.5-1 mm from the astrocytes strip. The coverslips were placed into a 6 well culture flask at 37°C and 5% CO_2. After 2 hours, the co-cultures were washed to remove unattached cells and 2ml/well of growth medium. This was changed every 48 hours and the cells were grown for 13 days when 5000 DRG neurons were seeded onto coverslips. Three independent co-culture experiments with six repetitions were carried out for each cell type. After 24 hours, co-cultures were fixed with 4% (w/v) PFA and immunostained to assess the distribution of cellular territories and neurite outgrowth. In addition, 14-day-old co-cultures of astrocytes with Schwann cells or ASC were collected from three wells for RT-PCR analysis.

1.6. *In vitro* angiogenesis assay

For Paper I, an *in vitro* angiogenesis assay kit (Millipore) was used for evaluation of tube capillary-like formation by endothelial cells. When cultured on ECMatrix™, a solid gel of basement proteins prepared from the Engelbreth Holm–Swarm mouse tumour, endothelial cells rapidly align and form hollow tube-like structures. ECMatrix was prepared in 96-well plates as described by the manufacturer, and 5 x 10^3 human umbilical vein endothelial cells (HUVEC; Invitrogen) were plated onto the surface in the presence of CM from ASC. Since we found that factors used to stimulate ASC interfered with the assay, we first stimulated the ASC for 24 h, then washed the cells four times with HBSS to remove stimulating factors, and reapplied regular growth medium for 48 h. HUVECs were allowed to attach for 4 h before tube formation was evaluated by light microscopy. Four random fields from each well were captured and analysed for total network length (continuously joined end-end cells) and the number of closed polygon-shaped structures. These values were used as a semi-quantitative measure of angiogenesis as recommended by the manufacturer. The experiments were repeated using ASC from different patients.

2. Experimental animals and surgery

Transplantation experiments were performed on adult (10–12 weeks) female Sprague–Dawley rats (Taconic Europe A/S). The animal care and experimental procedures were carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and also approved by the Northern Swedish Committee for Ethics in Animal Experiments (No. A36-12 and No. A186-12). All surgical procedures were performed under general anaesthesia using a mixture of ketamine (Ketalar®, Parke-Davis; 100mg/kg i.v.) and xylazine (Rompun®, Bayer; 10mg/kg i.v.). After surgery, the rats were given the

analgesic, Finadyne (Schering-Plough, Denmark; 2.5mg/kg, s.c.), normal saline (4ml, s.c.) and benzylpenicillin (Boehringer Ingelheim; 60mg, i.m.). Each animal was housed alone in a cage after surgery and exposed to 12-hour light/dark cycles, with free access to food and water.

2.1. Peripheral nerve injury

Under an operating microscope, the sciatic nerve was exposed and then divided 5mm below the exit point from sciatic notch and 5mm of sciatic nerve distal to the division was removed creating a 10mm gap. The 14mm long fibrin conduit (see below) was inserted in the gap, allowing for intubation of the proximal and distal nerve ends 2mm into the conduit. The conduits with ASC (n = 7) were fixed to the epineurium with three 10/0 Ethilon sutures at each end. The wound was then closed in layers. The control group comprised fibrin conduits containing fibrin matrix alone (no cells; n = 7). Animals were allowed to survive for 2 weeks and were treated daily with cyclosporine A (CsA, Sandimmun; Novartis) that was injected subcutaneously at 15mg/kg body weight, administered starting from 24 h before surgery.

Tubular fibrin conduits were moulded from two compound fibrin glue (Tisseel^{TM} Duo Quick; Baxter). All components were mixed under sterile conditions, and a silicone mould with a centrally placed metal rod was used to prepare tubular 14-mm-long conduits with uniform 1-mm-thick walls and 2mm lumen as previously described[50]. After glue polymerization, the rods and silicone mould were removed, and fibrin glue conduits were loaded with fibrin matrix with or without ASC. Fibrin matrix was produced by modifying the fibrin glue as previously described [60]. ASC or stimulated ASC cultured in parallel for 2 weeks were re-suspended with the diluted fibrinogen solution (2 x 10^6/20 μL). The diluted thrombin solution (20 μL) was injected into the lumen of a conduit and then, immediately the cell/fibrinogen suspension was added. The matrix was allowed to polymerize before surgical transplantation into the sciatic nerve defect.

2.2. Spinal cord injury

Dorsal cervical laminectomy was performed over C3-C4 up to the midline on the left hand side, by visualising the posterior spinal vein. Using an operating microscope, the dura mater was cut longitudinally over the left side of the spinal cord using microscissors. The DRGs of C3 and C4 were identified and a 23 gauge sterile needle was introduced at the dorsal root entry zone until the anterior surface of the vertebral canal was palpable with the needle tip. A lateral transection was made using the canal created and extent of injury confirmed using the needle tip and micro forceps. Immediately after the injury, the animals were randomly subdivided into three groups: (i) spinal cord injury (SCI) followed by injection of α-MEM alone and daily treatment with cyclosporine A (CsA) for 3 weeks (n=18), (ii) SCI followed by transplantation of ASC in α-MEM and daily treatment with CsA for 3 weeks (n=21) and (iii) SCI

followed by transplantation of ASC in α-MEM without CsA (n=5). Twelve normal uninjured rats served as baseline controls.

For transplantation, the cells were detached with trypsin/EDTA, washed and concentrated to $5x10^4$ cells/µl in α-MEM. After transfer into a siliconised glass micropipette (outer diameter 100µm) attached to a 5µl Hamilton syringe, 1.5µl of the cell suspension (75,000 cells) were injected slowly over 10 minutes into the lateral funiculus at a depth of 1mm (after confirming the micropipette tip was not resting on the anterior wall of the vertebral column), approximately 1mm cranial and 1mm caudal to the lesion site using a Stoelting's Lab Standard Stereotaxic Instrument (Stoelting Co., USA). The micropipette was left in place for an additional 2 minutes. The dura mater was covered with sterile saline soaked sponge (Spongostan®) to prevent dehydration, and the muscles and skin were closed in layers. Rats in groups (i) and (ii) then received the first dose of CsA (Sandimmum, Novartis; 15mg/kg s.c.), which continued for 3 weeks postoperatively, as previously described [61].

3. Tissue processing and analysis

3.1. Tissue harvest

Cultured cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH = 7.4) at room temperature for 20 minutes. The animals were deeply anaesthetized with an intraperitoneal overdose of sodium pentobarbital. For RT-PCR and Western blotting, spinal cord segments and corresponding DRGs were removed and frozen immediately in liquid nitrogen. For immunohistochemistry in Paper II, the rats were transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) and then cervical spinal cord segments C2, C3-C5 and C6 were removed, post-fixed in the same fixative for 2-3 hours, cryoprotected in 10% (w/v) and 20% (w/v) sucrose for 2-3 days and frozen in liquid isopentane. Serial transverse and longitudinal 16µm thick sections were cut on a cryomicrotome (Leica Instruments, Germany), thaw-mounted onto SuperFrost®Plus slides, dried overnight at room temperature and stored at -80°C before immunostaining.

3.2. RT-PCR

RT-PCR was performed to qualitatively measure mRNA expression levels. Total RNA was isolated from ASC using an RNeasy ™ kit (Qiagen, Sweden) and 1ng RNA incorporated into a One-Step RT-PCR kit (Qiagen) per reaction mix for the initial individual patient cell characterisation experiments (See Paper II, Figure 2), 10ng RNA extracted from tissue and transplanted cells (See Paper II, Figure 3) or 1 ng RNA was isolated from human ASC (passages 2–4) to compare neurotrophic and angiogenic expression profiles (See Paper I, Table 1). A thermocycler (Biometra, Germany) was used with the following parameters: a reverse transcription (RT) step (50°C, 30min) and a nucleic acid denaturation/RT inactivation step (95°C, 15min)

followed by 28-34 cycles of denaturation (95°C, 30sec), annealing (30sec) and primer extension (72°C, 1min) followed by a final extension incubation (72°C, 5min). Forward and reverse primer (all 5'→ 3') pairs (SigmaAldrich) with annealing temperatures used are listed in Paper II, Table 1. 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 ultraviolet (UV) illumination following GelRed™ nucleic acid stain (Bio Nuclear, Sweden) incorporation into the agarose.

3.3. qRT-PCR

For Paper I, 1ng of total RNA per reaction was converted into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoFast™ EvaGreen supermix (Bio-Rad) in a CFX96 Optical Cycler and analysed using the CFX96 manager software (Bio-Rad). Primers were manufactured by Sigma (See paper I, table 1), and reactions were optimized and processed according to the manufacturer with initial denaturation/DNA polymerase activation at 95°C for 30 s followed by PCR: 95°C for 5 s, variable annealing temperature (See paper I, table 1) for 5 s, and 65°C for 5 s repeated for 40 cycles. 18S was used as a housekeeping gene. Data were calculated as relative expressions according to the $\Delta C_{(t)}$ principle.

3.4. Western blotting

C3 spinal cord segments from the injured side were homogenised in lysis buffer containing 5mM ethylene glycol tetra acetic acid (EGTA), 100mM 1,4-Piperazinediethanesulfonic acid (PIPES), 5mM MgCl2, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100 and protease inhibitor cocktail (SigmaAldrich). Protein levels were then determined using the DC Protein Assay (BioRad, Sweden). Either ten or twenty micrograms of protein were loaded per lane onto 6% (v/v), 10% (v/v) or 15% (v/v) sodium dodecyl (SDS)-polyacrylamide gels and resolved at 200V. Following electrophoresis, the protein was transferred to PVDF membranes (80V for 75min or 30V for 90min) and then blocked in 5% (w/v) non-fat milk in Tris-buffered saline with Tween (TBS-T) for 1hour. The primary antibodies were diluted in the blocking solution and incubated with membranes overnight at 4°C (See Paper II, table 2). After 6x5 minute washes in TBS-T, secondary rabbit IgG (1:2000) or mouse IgG (1:1000) HRP-linked antibodies (both from Cell Signalling Technology, USA) were applied for 1 hour at room temperature (see Paper II, table 2). Finally, the membranes were washed for 6 x 5 minutes in TBS-T and the blots exposed to enhanced chemiluminescence reagent (GE Healthcare, Sweden) and developed onto Kodak XPS films. To ensure equal protein loading of samples, the membranes were stripped of antibody using 100mM glycine (pH 2.9) and processed for blotting with mouse anti-β-actin (1:20000; Millipore, USA). Films were scanned using an Epson Photoscanner and analysed using Scion Image (Scion Corporation, Maryland, USA), which performs peak area integration to determine the area of each band in pixel units. The

optical density of each protein was expressed as a ratio of the corresponding signal for β-actin.

3.5. Enzyme-linked immunosorbant assay (ELISA)

For paper I, ASC (1.25 x 10^5) were seeded into 96-well plates and maintained for 48 h. The supernatant was then collected and analysed by ELISA using the ChemiKine™ BDNF sandwich ELISA kit (Millipore) or NGF, GDNF, angiopoietin-1, and VEGF-A sandwich ELISA kits (RayBiotech, Inc.) according to the manufacturer's protocol. All samples were analysed in triplicate, and the absorbance was measured at 450nm on a SpectraMax190 microplate reader (Molecular Devices, Inc.). The quantity of neurotrophic and angiogenic factors (pg/mL) was calculated against standard curves that were produced using recombinant proteins provided in the kits and normalized to the final number of cells counted after 48 h of incubation.

3.6. Immunostaining

Immunostaining was performed on cells cultured on Lab-Tek® slides and coverslips, and longitudinal 16-mm-thick sections of fibrin conduits and spinal cord. After blocking with normal serum, the primary antibodies were applied for 2 hours at room temperature. In Paper I, the following primary antibodies were used: mouse anti-βIII-tubulin (1:500; Sigma-Aldrich), mouse anti-human nuclei (1:100; Millipore), rabbit anti-S100 protein (1:2,000; Dako), or mouse anti-RECA-1 (1:25; Abcam). Primary antibodies used in Paper II are listed in Table 2. After rinsing in PBS, secondary goat anti-mouse and goat anti-rabbit antibodies Alexa Fluor® 350, Alexa Fluor® 488 and Alexa Fluor® 568 (1:100-1:300; Molecular Probes) were applied for 1 hour at room temperature in the dark. The slides were coverslipped with ProLong mounting media containing 4'-6-diamido-2-phenylindole (DAPI; Invitrogen Life Technologies, Sweden). The specificity of staining was tested by omission of the primary antibodies.

3.7. Analysis of regeneration in fibrin conduits

Axon regeneration distance was measured using an optical microgrid; the length was measured from the beginning of the proximal nerve stump to the last visible sprout of the regenerating front. Every sixth to eighth section was first scanned under the microscope to identify the sections containing the longest axons, and then, four sections within the selected range were used for analysis. In addition, beginning at 2mm distal to the end of the proximal stump (where individual axons could be distinguished), axonal profiles were counted in a line perpendicular to the direction of the conduit in serial, high power fields distally every 500 mm until the field contained no more positive β-III-tubulin staining. The number of RECA-1-positive capillary structures was also counted from eight random fields/slides obtained from the mid-point of the conduits.

3.8. Analysis of regeneration in spinal cord

Serotonin-positive raphaespinal axons were quantified in the ventral horn of the C2 and C6 spinal segments and glial fibrillary ancillary protein (GFAP)-positive astrocytes, C3bi complement receptor expressing (OX42)-positive microglial cells and rat endothelial cell antigen (RECA)-1-positive vascular endothelium were studied in the lamina VII of the C2 and C3 segments at 8 weeks after SCI and ASC transplantation. All images were captured at 400x final magnification with a Nikon DS-U2 digital camera. The relative tissue area occupied by immunostained profiles was quantified in 10 randomly selected sections in 50x50 µm areas (18.9 pixels per 1µm tissue length) for serotonin-positive axons and 150x150 µm areas (3.8 pixels per 1µm tissue length) for astrocytes, microglial cells and vascular endothelium using Image-Pro Plus software (Media Cybernetics, Inc., USA).

4. Image processing

For preparation of figures, the captured images were resized, grouped into a single canvas and labelled using Adobe Photoshop CS4 software. The contrast and brightness were adjusted to provide optimal clarity.

5. Vertical cylinder exploration test

For Paper II, forelimb asymmetry was measured using vertical exploration of a clear plastic cylinder to assess the extent of motor recovery of the forelimb paws, as described previously [62, 63]. The equipment was constructed from clear Plexiglas and mirrors placed to ensure paw placement was captured from every angle. Briefly, this used a rectangular base (40 x 50 cm), with pillars placed at each corner (16 cm high). On top of this was placed a cylinder (External diameter 20 cm, height 21 cm) with three mirrors on stands (25 x 30 cm) around it to allow 360° views of the base and sides whilst the animal explored the cylinder. A video camera (Canon Legria HF R206 3.28mpix 20x optical zoom) was placed on a tripod 1 metre from the centre of the cylinder. The animal was placed within the cylinder and filmed immediately for 3 minutes. Exploration of the vertical cylinder was assessed (one episode of exploration was deemed to be the forepaw resting on the surface for >0.5s when measured on playback programme). The number of episodes of exploration using the injured and uninjured forepaws were counted and calculated as a percentage (number of episodes of injured use/number of episodes of uninjured use x100). Testing was performed at 3, 5, 7 and 9 weeks postoperatively.

6. Statistical analysis

One-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls Multiple Comparison Test was used to determine statistical differences between the experimental groups (Prism®, GraphPad Software, Inc; USA). Statistical significance was set as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

1. Human ASC in culture

1.1. ASC characterisation

Cultured ASC displayed variable morphology ranging from the flat fibroblast-like cells to spindle-shaped cells. Immunostaining revealed that the cells at passages 2-6 were negative for haematopoietic markers CD14 and CD19 and positive for stromal/stem cell markers CD54, CD90, CD105, CD146, HCAM (CD44) and for the extracellular matrix molecules fibronectin and laminin (See Paper I, Fig. 1 and Paper II, Fig. 1). The cells were also able to differentiate into adipocytes and osteoblast-like cells. The ASC from different patients expressed varying levels of transcripts for several neurotrophic and angiogenic factors (See Paper II, Fig. 2) including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell derived neurotrophic factor (GDNF), vascular endothelial growth factor-A (VEGF-A), angiopoietin-1 (Ang-1).

1.2. Stimulation of ASC enhances neurotrophic properties *in vitro*

Stem cells were stimulated with a mixture of growth factors (forskolin, FGF-2, PDGF-AA, and neuregulin1-β1) for a period of 2 weeks. Semi-quantitative RT-PCR analysis showed that the stimulation protocol enhanced the expression of the neurotrophic factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell derived neurotrophic factor (GDNF) but down-regulated the expression of neurotrophin 3 (NT3) (See Paper I, Fig. 2A). ELISA analysis indicated that increased mRNA levels correlated with enhanced secretion of BDNF and GDNF protein (See Paper I, Fig. 2B). Stimulation of the cells resulted in the release of 101.70±14.45 pg/mL BDNF and 18.79±3.27 pg/mL GDNF, both of which were significantly ($P < 0.01$) higher than the corresponding protein levels secreted by unstimulated cells. The expression levels of the angiogenic molecules, vascular endothelial growth factor-A (VEGF-A), and angiopoietin-1 were also increased (See Paper I, Fig. 2C). Secreted angiopoietin-1 levels were significantly ($P < 0.001$) increased from 5.11±0.77 ng/mL in unstimulated cells to 17.63±1.33 ng/mL in stimulated cells (See Paper I, Fig. 2D). Unstimulated ASC released high levels of VEGF-A (20.92±3.54 ng/mL), but this could also be further increased with the stimulation protocol (See Paper I, Fig. 2D). Next, *in vitro* assays for neurite outgrowth and angiogenesis were used to determine whether the increased levels of the growth factors had some functional consequences. Rat DRG neurons were seeded onto coverslips and after 48 h, they were immunostained with β-III-tubulin antibody to measure neurites. Neurons seeded in medium conditioned by ASC or stimulated ASC extended a number of long neurites that were absent in the DRG neuron cultures exposed to the respective media controls alone (See Paper I, Fig. 3A). Computerized image analysis showed that the average longest neurite and the total neurite outgrowth

(mm) were significantly higher in neurons treated with stimulated ASC conditioned media compared with ASC conditioned media (See Paper I, Fig. 3B,C).

1.3. Stimulation of ASC enhances angiogenic properties *in vitro*

Angiogenesis was assessed by measuring the formation of capillary tube-like formations of endothelial cells (HUVEC) on a nutrient-rich extracellular matrix gel (See Paper I, Fig. 4). HUVEC seeded in stem cell growth medium adopted an elongated phenotype within 4 h, which was in contrast to cells grown on tissue culture plastic that showed a characteristic "cobblestone" morphology (See Paper I, Fig. 4A). When the HUVEC were exposed to CM from ASC or stimulated ASC, there was a noticeable increase in the amount of elongation and the formation of more complex networks of cells (See Paper I, Fig. 4A). Quantification of the length of the tubes (by measuring the continuously linked end-to-end cell contacts) showed there was a significant ($P < 0.05$) increase from 1,696±192.30 mm in control cultures to 2,346±186.30 mm in HUVEC exposed to ASC medium and a further increase to 3,667±172.50 mm in the presence of stimulated cells CM (See Paper I, Fig. 4B). HUVEC cultured in medium from both types of stem cells also showed significant increases in the number of closed networks formed when compared with control medium alone (See Paper I, Fig. 4C). The potentiaton of the angiogenic response in the presence of stimulated-ASC medium was not attributable to the stimulation factors alone, as medium was collected from the cells after these molecules had been washed out (see Materials and Methods section).

1.4. Confrontation assay of astrocytes with ASC and Schwann cells

Confrontation culture assays demonstrated relatively limited intermingling of cortical astrocytes with both ASC and Schwann cells after 13-14 days *in vitro* (See Paper II, Fig. 3A,B). To investigate how the interaction of astrocytes with Schwann cells or ASC could influence axonal growth *in vitro*, we applied 5000 adult DRG neurons along the border between the interacting cells for 24 hours. In the co-cultures of astrocytes and Schwann cells, most neurons that landed on the interface between two cell territories grew their axons in close association with Schwann cells (See Paper II, Fig. 3A). The axons were also found to leave a group of Schwann cells, cross over astrocyte surfaces and then continue to grow on adjacent Schwann cell territory. In contrast with co-cultures of astrocytes and Schwann cells, in co-cultures of astrocytes and ASC, the neurons had no preferential growth and axons were found on both cell types regardless of their initial attachment (See Paper II, Fig. 3B). Consistent with these observations RT-PCR analysis of the cultures revealed significant activation of astrocytes in co-cultures of astrocytes and Schwann cells when compared with co-cultures of astrocytes and ASC (See Paper II, Fig.3C). Thus, we found increased transcript levels for glial fibrillary acidic protein (GFAP), growth inhibitory CSPGs, neurocan and aggrecan, extracellular matrix molecule tenascin C, pro-inflammatory cytokine TGF-β and adhesion molecule β1integrin (See Paper II, Fig. 3C).

2. Transplantation of human ASC after peripheral nerve injury

2.1. Effects of ASC on regeneration in fibrin conduits after nerve injury

A 10mm rat sciatic nerve injury model was used to assess the in vivo effects of the cells. Fibrin conduits were seeded with 2 x 10^6 cells and nerve regeneration was measured 2 weeks after transplantation. Using β-III-tubulin immunofluorescence to stain axons (See Paper I, Fig. 5A). The counts of regenerating axons between the proximal stump and regenerating front were significantly higher ($P < 0.05$) in the stimulated-ASC group compared with rats treated with the conduit filled with matrix alone and devoid of cells (See Paper I, Fig. 5B). There was also a significant increase in the distance of the furthest regenerating axons in both stem cell-seeded groups (See Paper I, Fig. 5C). There was no significant difference between these two cell groups. Staining with a human nuclear antigen-specific antibody showed the ASC were still present in the conduit at 2 weeks (See Paper I, Fig. 5D).

2.2. Effects of ASC on the retrograde reactions in DRGs and spinal cord

qRT-PCR analysis of the sensory L4-L6 DRG neurons and corresponding motor neuron containing spinal cord segments revealed that a number of regeneration- and apoptosis-related genes were differentially modulated after the nerve repair (See Paper I, Fig. 6). Compared with control uninjured animals, the rats treated with the fibrin conduits without cells showed an approximately fourfold increase in GAP-43 expression levels in the DRG and spinal cord. Addition of stem cells to the nerve conduits had no further effect on GAP-43 expression in the DRG but significantly increased the expression levels in the spinal cord (See Paper I, Fig. 6A). There was a 3.50±0.26-fold increase in caspase-3 expression levels in the DRG of nerve-injured animals treated with conduits without cells compared with control rats (See Paper I, Fig. 6B). DRG caspase-3 levels were significantly ($P < 0.01$) reduced when the conduits were filled with stimulated cells, while unstimulated ASC had no significant effect (See Paper I, Fig. 6B). There were no significant differences in caspase-3 expression levels in the spinal cord of all four groups (See Paper I, Fig. 6B). Compared with control uninjured animals, activating transcription factor 3 (ATF-3) expression levels were significantly ($P < 0.001$) increased in both the DRG and spinal cords of rats with nerve injury repaired with conduit alone (See Paper I, Fig. 6C). Addition of either cell type to the conduits further significantly increased the expression of ATF- 3 in the spinal cords, but there was no effect on levels in the DRG (See Paper I, Fig. 6C). The binding partner of ATF-3, c-jun, was significantly increased 3.68±0.44-fold above control levels in the DRG of nerve-injured animals treated with conduits without cells (See Paper I, Fig. 6D). These levels were significantly ($P < 0.05$) reduced in the animals treated with nerve conduits containing either type of stem cell (See Paper I, Fig. 6D). Animals with nerve injury and repair in

all three groups showed significantly ($P < 0.001$) reduced c-jun expression levels in the spinal cords compared with control rats (See Paper I, Fig. 6D).

2.3. Effects of ASC on angiogenesis

Angiogenesis within the nerve conduits was determined using immunofluorescence staining with rat endothelial cell antigen (RECA-1) antibody (See Paper I, Fig. 7A). At the mid-point of the conduits containing fibrin matrix alone (no cells), there were a few RECA-1-positive cells, but in the conduits containing ASC and stimulated ASC, there was a marked increase in reactivity. Quantification of the number of RECA-1-positive cells showed a significant increase from 9.70 – 5.63/mm2 in conduits without cells to 26.49±3.66/mm2 ($P < 0.05$) in ASC seeded conduits and 34.55±4.86/mm2 ($P < 0.01$) in stimulated ASC-loaded conduits (See Paper I, Fig. 7B).

3. Transplantation of human ASC into injured spinal cord

3.1. Survival of transplanted ASC

One week after SCI and ASC transplantation with CsA treatment, numerous cells immunolabelled for human nuclear antigen (HNA; See Paper II, Fig. 4A) were found in the injection sites cranial and caudal to the lesion site. The injection sites were connected to the trauma zone by narrow tracts formed by transplanted ASC. At 2 weeks postoperatively, the number of HNA-labelled ASC were noticeably reduced (See Paper II, Fig. 4B) and at 3 weeks, only single HNA-labelled cells were found in the trauma zone and injection sites (See Paper II, Fig. 4C). However, a significant number of surviving ASC were found in the anterior median fissure of the spinal cord, around the blood vessels (See Paper II, Fig. 4C). There was no migration of HNA-labelled cells away from the transection site along the cranio-caudal axis of the spinal cord. Additional immunostaining with antibodies against GFAP revealed numerous migrating astrocytes into the trauma zone and injection sites (See Paper II, Fig. 4A-C).

3.2. Expression of human-specific growth factors by ASC transplants

RT-PCR analysis using human selective primer sequences showed that transplanted ASC continued to express mRNA for neurotrophic and angiogenic factors NGF, BDNF, FGF-2, VEGF-A and Ang-1 one week after transplantation (See Paper II, Fig. 4D). Expression of VEGF-A and FGF-2 was seen also at 2 and 3 weeks postoperatively. There were no detectable levels of human growth factors at 2 weeks after SCI alone (confirming the human sequence selectivity of the primers) and after SCI followed by transplantation of ASC without CsA treatment (See Paper II, Fig. 4D).

Immunostaining of spinal cord sections for BDNF and FGF-2 confirmed expression of these growth factors in the ASC injection sites at 1 week postoperatively (See Paper II, Fig. 5A,B). The labelling was significantly decreased 2 weeks after ASC

transplantation (See Paper II, Fig 5D,E). After transplantation, ASC continued to express laminin both at 1 week and 2 weeks postoperatively (See Paper II, Fig. 1I and Fig. 5C,F). In addition, laminin immunostaining revealed increased ingrowth of blood vessels into transplantation sites. At 1 week after injury, growth inhibitory CSPG neurocan was found predominantly around the transplantation sites (See Paper II, Fig. 5G,H) but at 2 weeks postoperatively it was increased throughout C3-C4 spinal cord segments (See Paper II, Fig. 5I,J). However, the pattern of neurocan expression around ASC injection sites was changed when compared with 1 week transplantation site (See Paper II, Fig. 5 G and Fig. 5I). Thus, GFAP-positive astrocytes migrating into injection sites were not always associated with areas of increased neurocan immunoreactivity (See Paper II, arrows in Fig. 5I).

To assess whether expression of growth factors by transplanted ASC could possibly affect the total protein levels of these factors in the spinal cord tissue, we performed Western blot analysis of the C3 spinal cord segment rostral to the injured side at 2 weeks postoperatively. The results demonstrated that transplanted ASC did not change the total protein levels of BDNF, VEGF and FGF-2 (See Paper II, Fig. 6). Analysis of extracellular matrix molecules laminin and neurocan also did not reveal any significant differences between spinal cord injury and ASC transplantation groups (See Paper II, Fig. 6).

3.3. Effects of ASC transplantation on axonal regeneration

At 8 weeks after SCI with CsA treatment, single serotoninergic 5HT-positive raphaespinal terminals regenerated approximately 100-150µm into the trauma zone (SCI; See Paper II, Fig. 7). Transplantation of ASC with CsA promoted extensive ingrowth of raphaespinal axons into the trauma zone at 8 weeks postoperatively with single terminals entering the distal spinal cord for at least 200-300µm (SCI+ASC; See Paper II, Fig. 7).

Quantification of 5HT-positive axonal arborisations in the ventral horn of the C2 cervical segments cranial to the lesion site revealed that SCI with CsA treatment induced an almost four-fold increase in the density of raphaespinal terminals on the side of injury ($P<0.001$; See Paper II, Fig. 7F) but had no effect on the contralateral side (See Paper II, Fig. 7G). Transplantation of ASC reduced the sprouting of raphaespinal terminals on the side of injury by approximately 45% when compared with SCI ($P<0.001$; See Paper II, Fig. 7F) and, at the same time, stimulated a greater than four-fold increase in terminal density on the contralateral side ($P<0.001$; See Paper II, Fig. 7G).

In the cervical C6 segments below the lesion site, SCI alone reduced the density of raphaespinal terminals by 78% and 70% on the side of injury and the contralateral side, respectively ($P<0.001$; See Paper II, Fig. 6H,I). Transplantation of ASC induced

a three-fold increase in the density of terminals on the side of injury ($P<0.001$; See Paper II, Fig. 7H) and two-fold increase on the contralateral side ($P<0.01$; See Paper II, Fig. 7I) when compared with SCI. However, cell transplantation did not recover the density of raphaespinal innervations in the ventral horns below the injury when compared with control uninjured animals (CONT; $P<0.001$; See Paper II, Fig. 7H,I).

3.4. Effects of ASC transplantation on glial cell reactions and vascular endothelium

At 8 weeks postoperatively, the spinal cord trauma zone was surrounded by a dense network of astrocytic processes (See Paper II, Fig. 8A). Transplantation of ASC cranial and caudal to the site of injury changed the structure of the astroglial scar (See Paper II, Fig. 8B). Numerous GFAP-positive astrocytic processes extended into the trauma zone in a chain-like pattern and were associated with the regenerating raphaespinal axons (See Paper II, Fig.8C). Analysis of the glial cell reaction cranial to the lesion site revealed that SCI combined with CsA treatment resulted in a three to four-fold increase in immunoreactivity for GFAP-positive astrocytes ($P<0.001$; See Paper II, Fig. 9A and Fig. 8D, E) and OX42-positive microglia ($P<0.001$; See Paper II, Fig. 9B and Fig. 8G, H). In addition, SCI increased the density of RECA-1 positive vascular endothelium by 60% (See Paper II, Fig. 9C). Transplantation of ASC significantly attenuated the astroglial and microglial cell reactivity ($P<0.001$; See Paper II, Fig. 9A, B) but had no effect on the vascular endothelium ($P<0.05$; See Paper II, Fig. 9C).

3.5. Effects of ASC on forelimb paw motor recovery

Analysis of the forelimb paw motor recovery with vertical cylinder exploration test demonstrated that there were no significant differences between the two experimental groups at any time point (3, 5, 7 or 9 weeks), postoperatively ($P>0.05$; See Paper II, Fig. 10).

DISCUSSION

A number of different mechanisms have been proposed regarding the role stem cells have after transplantation [4, 64, 65]. These include reduction of inflammation or gliosis and creation of a growth promoting environment; enhanced preservation of host neuronal and glial cells; restoration of neuronal circuitry; increased expression of neurotrophins/cytokines by the transplanted or host cells; promotion of angiogenesis; replacement of myelinating cells or neurons; remyelination of spared axons; bridging of cysts or cavities (acting as a bioactive scaffold) and stimulation of endogenous precursor cells. In this thesis, we sought to determine through which mechanisms ASC would exert their effects in our experimental models.

1. Production of growth factors by human ASC

In both studies we found that the ASC were able to produce a range of neurotrophic and angiogenic factors *in vitro*. Stimulation of the ASCs resulted in increased NGF, BDNF, GDNF, VEGF-A, and angiopoietin-1 gene expression and consequent enhanced levels of these secreted proteins. Cells with the broadest trophic factor expression profiles were transplanted *in vivo*, where production continued but declined over 3 weeks post-transplantation with immunosuppression.

We have previously demonstrated the beneficial effects of BDNF in spinal cord injury when applied via an osmotic pump [66] or when produced by transplanted bone marrow stem cells (BMSC) [67]. BDNF has been described to induce plasticity in the injured spinal cord and plays a role in axonal myelination [68-70]. FGF-2 has been shown to protect CNS neurons, reducing the effects of anoxia, hypoglycaemia, excitatory amino acids, free radicals, excess intracellular calcium, and nitric oxide [71-74], thereby decreasing the need for macrophage/microglia recruitment. Importantly, exogenously applied FGF-2 has shown to lead to physical alignment of astrocytes [75]. As well as the angiogenic effects of VEGF-A, it has also been shown to exert neuroprotective effects when injected into the injured tissue, by reducing retrograde degeneration, apoptosis and spinal cord tissue loss [76, 77].

Other cell types such as neural precursor cells (NPC) have been considered suitable for transplantation into the injured spinal cord. In addition to their ability to differentiate into neurons and glial cells they express a wide range of neurotrophic factors. It was shown *in vitro* that NPC and bone marrow MSC have unique expression patterns for various trophic factors, which is to some extent dependent on culture conditions [78]. In another interesting study by the same research group the *in vivo* trophic factor expression levels produced by transplanting NPCs or bone marrow MSC was compared [79]. They found increased expression of ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), and FGF-2 by neural precursor cells

following SCI, compared to bone marrow MSC, which only expressed NGF, LIF, and IGF-1 [79]. Many of the trophic factors investigated in these studies have been shown to be expressed by ASC and are also likely to be influenced by transplantation into the injured spinal cord. However, other studies report that bone marrow MSC produce also BDNF, VEGF and GDNF [80-83]. Comparisons have shown that ASC produce significantly higher amounts of growth factors like VEGF, HGF and BDNF when compared to bone marrow MSC [84-86].

In Paper I we demonstrated that growth factor stimulation of the ASC resulted in increased NGF, BDNF and GDNF gene expression and consequent enhanced levels of these secreted proteins. The stimulating mixture contained forskolin, FGF-2, PDGF-AA and neuregulin-1 and these factors may act alone or together to control the signalling pathways mediating the expression of these neurotrophic factors. For instance, forskolin increases NGF mRNA levels in Schwann cells [87] whilst FGF-2 enhances BDNF expression in retinal ganglion cells [88]. Previously we showed that the same mixture of factors could induce the expression of glial cell proteins in rat ASC [55]. The elevated secretion of neurotrophic factors was consistent with increased neurite outgrowth of DRG neurons exposed to conditioned medium from the stem cells. We have previously reported that human bone marrow MSC also enhance DRG neurite outgrowth but most likely independently of BDNF [89]. Other studies have shown that human ASC express a range of neurotrophic factors which can enhance neurite outgrowth of neuronal cell lines [90-92].

Stimulation of ASC also enhanced the expression of the angiogenic molecules, VEGF-A and angiopoietin-1. Numerous studies have indicated that VEGF plays a pivotal role in ASC mediated regeneration [93]. When ASC are cultured under hypoxic conditions they secrete increased levels of VEGF and can convert to an endothelial cell phenotype [94, 95]. ASC are often genetically modified to over-express VEGF [96, 97] but we observed significant VEGF secretion levels in control cultures of ASC which could be further potentiated by treatment with stimulating factors. Paracrine release from VEGF engineered ASC was shown to promote endothelial cell survival and tube formation *in vitro* [96]. We found that unstimulated ASC could promote HUVEC capillary-like formation and that this was significantly enhanced by stimulating the stem cells. In contrast, the results of Strassburg et al showed that ASC conditioned medium had no effect on HUVEC but rather, direct cell contact of ASC with endothelial progenitors was necessary for *in vitro* angiogenesis [98]. The growth factors FGF-2 and PDGF used in our stimulation protocol are the most likely to control the production of VEGF since there is significant cross-talk between all three molecules [99]. In addition to the secretion of VEGF we found elevated levels of angiopoietin-1 in the stimulated ASC medium. We have previously shown that rat ASC express angiopoietin-1 [100] but there are few studies about this molecule in human cells. Blasi et al suggested that ASC can be distinguished from

dermal fibroblasts by their increased angiogenic profile which is partly attributable to expression of angiopoietins [101]. In contrast, another study showed that it was necessary to genetically modify the ASC to enable significant secretion of angiopoietin [102]. In the context of our study it is also interesting to note that adipocyte derived angiopoietin-1 supports neurite outgrowth and synaptogenesis of sensory neurons [103].

2. Effects of ASC on extracellular matrix molecules

There are also no previous reports showing stem cells acting by reducing chondroitin sulphate proteoglycan (CSPG) production. CSPGs form a peri-neuronal net and after SCI, can be found in close proximity to the scar, acting to impede axonal regrowth and so preventing synapse formation. We investigated the effects of ASC on neurocan expression, a CSPG produced by astrocytes, fibroblasts and microglia, with a peak production at 2 weeks [104] and found a decrease in neurocan immunostaining around the cell transplantation sites. We also observed significant sprouting and ingrowth into injury site of 5HT-positive axonal terminals. This is in line with a previous report that chondroitinase ABC digestion of the perineuronal CSPG net promotes functional collateral sprouting in the cuneate nucleus after cervical spinal cord injury [105]. It is also possible that 5HT sprouting was supported by continuous expression of laminin [106], which has been shown to aid serotonergic sprouting following spinal injury.

3. ASC promote axonal regeneration after peripheral nerve injury

Both control and stimulated ASC enhanced the distance of axon regeneration at 2 weeks following sciatic nerve injury and repair but the stimulated cells evoked more total outgrowth. The human nuclear antigen positive transplanted cells were detected in high abundance throughout the nerve conduits but only a few showed co-staining with S100 protein suggesting against significant *in vivo* differentiation towards a Schwann cell phenotype. Since our first report of Schwann cell-like differentiation of rat ASC [55] a number of research groups have investigated the effect of rat ASC on peripheral nerve regeneration. We showed that Schwann cell-like differentiated ASC could enhance motor neuron regeneration and improve evoked electrical potentials in the gastrocnemius muscle [50] and also at early stages of regeneration we observed a down-regulation of apoptotic markers in the DRG suggestive of a pro-survival effect [107]. Undifferentiated rat ASC have also been shown to enhance peripheral nerve repair [108, 109] and they may act synergistically with Schwann cells to secrete growth factors such as NGF [110]. Alternatively the stem cells can be neurally induced to release enhanced levels of BDNF, which is necessary for their nerve stimulatory effects [111]. This may also account for our improved results using stimulated human ASC.

There are significantly fewer reports using human ASC in animal peripheral nerve injury models. Marconi et al reported that human ASC systemically injected into a

mouse model of sciatic crush could accelerate functional recovery and this might be attributed to a reduced inflammatory reaction and ASC interaction with endogenous Schwann cells to produce enhanced levels of GDNF [112]. Adipose precursor cells transplanted in polycaprolactone conduits were shown to reduce muscle atrophy in a rat short nerve gap defect model but the improvements in the sciatic functional index were temporary [113]. Furthermore in that study there was no evidence of stem cell trans-differentiation *in vivo* [113] which is in contrast to the recent report by Tomita et al. [92] in which the authors had first stimulated the cells using the same protocol as described in our study. Repetitive stimulation of ASC with neural differentiation medium can also boost the therapeutic effects human ASC in the rat sciatic nerve injury model [114].

4. ASC stimulate axonal sprouting after spinal cord injury

Current literature suggests that CNS axons sprout in the region of the scar [115] in an attempt to bridge it [116], and thereby compensate for the degraded axons and neuronal bodies. This process has been shown to be enhanced with neurotrophic support from other cell types such as bone marrow MSC, ASC, neural crest stem cells and umbilical somatic stem cells [61, 67, 70, 79]. In our spinal cord injury model, we found ASC transplantation to significantly decrease sprouting of the descending serotonergic fibres in C3 segment on the injured side. We hypothesise that this may occur due to the cumulative effect of a number of factors. Firstly, improved survival of the cranially placed neurons and axons decreased the need for excessive sprouting as more area present. Secondly, decreased reactivity of the astrocytes and their realignment into chain-like structures allowed for relatively unhindered growth of the fibres into and across the transection site [75, 117, 118], as a result of decreased microglial activity, decreased neurocan production [119] and increased FGF-2 release [120]. Remodelling of the nervous system occurred with contralateral sprouting of the serotonergic axons at C2 spinal cord level possibly through the decreased CSPG production [105] and retrograde transport of growth-promoting molecules. We found the combined effect is an increase in serotonergic fibres below the lesion at C6, on both the injured and uninjured sides, in keeping with recent findings described in the reticulospinal tract following SCI [121]. This pattern of plasticity in the serotonergic system has been shown to correlate with improved functional outcomes [122]. To the best of our knowledge, this is this the first time that this has been described with ASC, although similar effects have been shown after bone marrow MSC transplantation [123].

5. Effects of ASC on expression of regeneration-associated genes

Previous studies using human ASC in PNI have not elucidated the potential mechanisms by which the stem cells could directly act on the nervous system to enhance regeneration. We therefore examined the effect of stem cell transplantation on the expression of a number of genes known to be affected by peripheral nerve

injury. Growth associated protein GAP-43 is a molecule up-regulated in sciatic motor neurons and L4-L6 DRG neurons following sciatic nerve injury [124] and has been implicated as one of several important mediators of peripheral nerve regeneration [125]. Typically treatments such as electrical stimulation and growth factor administration which stimulate regeneration are associated with elevated levels of GAP-43 [126, 127]. We found that nerve repair performed with either ASC or stimulated ASC increased the expression levels of GAP-43 in the spinal cord but not the DRG. This suggests that the stem cells might enhance motor neuron regeneration to a greater extent than sensory neurons. A number of studies have shown that peripheral nerve axotomy leads to DRG sensory neuron cell death [128, 129]. In this study we found that using stimulated human ASC we were able to reduce the expression levels of caspase-3, an important mediator of apoptosis, suggesting cell transplantation could provide a neuroprotective effect in addition to boosting regeneration. This is consistent with our previous study using rat ASC [107] and another report using a mouse model [130]. Regeneration and apoptosis associated genes are controlled by multiple signalling pathways and in this study we examined the expression levels of ATF-3 and c-jun. ATF-3 is rapidly induced in DRG sensory neurons and motor neurons following axotomy [131]. ATF-3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration [132] and in the central nervous system, regenerating neurons are associated with increased ATF-3 expression [133]. Stem cell transplantation increased ATF-3 expression in the spinal cord but not DRGs, consistent with the GAP-43 regeneration gene expression profile. Interestingly, transgenic mice constitutively expressing ATF-3 do not show enhanced levels of GAP-43 [134] but GAP-43 expression levels are higher in ATF-3 positive neurons after electrical stimulation[135]. In the DRG it has been shown that non-regenerating neurons fail to up-regulate ATF-3 [136]. In our experiments it is possible that the stem cells boosted the number of regenerating motor neurons via enhanced expression of ATF-3.

ATF-3 can physically interact with another transcription factor, c-jun, to modulate the nervous system response to axotomy [137]. c-jun is one of the earliest molecules up-regulated following nerve injury and it has been shown to regulate diverse responses underlying degeneration, survival and regeneration [138]. This apparent dichotomy of function is highlighted by studies of c-jun deficient mice which show that lack of c-jun prevents neuronal cell death following facial nerve axotomy but impairs regeneration and reinnervation of targets [139]. Further studies in which neuron specific c-jun was deleted showed similar effects [140]. We found, consistent with previous reports [141], that c-jun was up-regulated in the injured DRG and when we transplanted stem cells these levels were reduced. Thus stem cells might exert a neuroprotective role (consistent with reduced caspase-3 levels) via release of molecules which act on c-jun. For instance, NGF which was up-regulated by our stimulation protocol has been shown to reduce injury-induced increases in c-jun

immunoreactivity [142]. Conversely, inhibition of c-jun phosphorylation has been shown to reduce axonal outgrowth of DRG neurons [143]. Similar anomalies have been reported when studying c-jun expression in the motor neurons. Increased levels of phosphorylated c-jun are observed in distally axotomised motor neurons of neonatal rats but this is not the case for adult rats [144]. However, avulsion injury in adult rats does result in increased phospho c-jun which is associated with significant cell death [144]. A long term association of phosphorylated c-jun with ATF-3 in axotomised motor neurons correlates with degenerating neurons [145]. In other studies, decreased c-jun expression has been correlated with impaired motor neuron regeneration in aged animals [146]. We found that nerve injury alone down-regulated c-jun levels in the spinal cord and we therefore propose that this might protect motor neurons against axotomy induced cell death. Nevertheless, how decreased c-jun levels can be correlated with our other observations of increased GAP-43 expression and improved regeneration in the periphery remains to be elucidated. It should be noted that we have examined these molecules at just one time point and the interaction between the various transcription factors and regeneration-associated genes is likely to be a very dynamic process following injury.

6. Effects of ASC on glial cell reactions

Astrocytes play a key role in the pathophysiology after SCI [147], leading to decreased production of growth factors and increased expression of inhibitory molecules [148]. Reduced astrocyte activation may occur as a result of early inactivation of microglia [117, 149], although the interaction is complex and reduced levels of CSPGs have been shown to decrease microglial cell activity [147]. In our study we demonstrated that ASC transplantation significantly reduced astrocyte activation 8 weeks post operatively. Similar effects have been described for bone marrow MSC and umbilical somatic stem cells [61, 67, 70].

Secondary damage in SCI is propagated by CNS glial cells and cells infiltrating from the peripheral circulation [150]. Together, they seal off the injured area, decrease pro-growth factors, and increase extrinsic inhibitory molecule production [115]. A recent report also demonstrates that blood derived macrophages can facilitate secondary axonal dieback after spinal cord injury [151]. Although our findings that ASC attenuated activation of OX42-labeled macrophage/microglial cells is not novel and has been shown for other cell types, it could contribute to increased sprouting of 5HT-positive axons.

7. Effects of ASC transplantation on angiogenesis

Many studies have demonstrated that vascularised nerve grafts are superior to non-vascularised nerve grafts with regard to healing in peripheral nerve injury. For example, the addition of VEGF to silicone conduits improves nerve regeneration [152] and nerve conduits that facilitate early vascularisation are superior to simple

silicone conduits [153]. Enhanced angiogenesis has been suggested to be one beneficial effect of MSC transplantation [76, 86, 154, 155]. VEGF-A and angiopoietin-1 released from ASC in SCI could decrease damage to the neurons and axons, preventing demyelination, which may reduce the need of the system to induce neovascularisation [76].

Our results in Paper I showing an increased amount of RECA-1-positive staining in conduits supplemented with ASC which could be attributed to the high levels of VEGF and angiopoietin secreted by the stem cells. Other very recent studies have indicated that human ASC used in animal experimental models can promote tissue formation and enhance graft retention as a result of enhanced vascularity [156, 157]. Our studies of ASC transplanted into the injured spinal cord in Paper II also demonstrated that ASC can produce angiopoietin-1 and VEGF-A for at least 3 weeks postoperatively. However, in contrast to peripheral nerve injury, we did not find any effect on neovascularisation after SCI at 8 week postoperatively.

8. Immunosuppressive treatment and human ASC transplantation

A number of studies have shown the survival of human MSC *in vivo* with [86, 158] or without [159, 160] immunosuppression. We found CsA was required for human cell survival, as we have previously reported when using human bone marrow MSC in the rat sciatic nerve injury model [60]. The loss of ASC is accelerated by recruitment of the adaptive immune response to aid innate immunity [161]. It has been shown that these effects are reduced when CsA is administered [162] thus, prolonging survival of the transplanted ASC, as seen in our experiments. This could reduce levels of ED1 positive macrophage/microglial cells as described by Shi et al. [163], who found that ASC supplement the immunosuppresive effects of CsA through Jagged-1/Notch-related inhibition of NF-κB signalling. Nevertheless in a non xenogenic setting, human ASC could act as effective suppressors of T cell proliferation [164] and B cell function [165] and thereby modulate the inflammatory reaction after injury. Interestingly human ASC can also facilitate the immunosuppressive effects of CsA [166] so could potentially be used to reduce the adverse effects of CsA which is a requirement for effective nerve allografting.

CONCLUSIONS

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

- Human ASC express various neurotrophic and angiogenic molecules, do not activate astrocytes in culture and promote neurite outgrowth from adult rats sensory DRG neurons. Stimulation of human ASC with a cocktail of growth factors leads to enhanced expression of neurotrophic and angiogenic molecules.

- Stimulated ASC boost axon regeneration and angiogenesis when transplanted within a nerve conduit to repair a peripheral nerve injury.

- After transplantation into injured spinal cord, ASC can survive for 3 weeks and continue to express growth factors when combined with CsA treatment. Transplanted ASC induce sprouting of serotonergic raphaespinal axons, reorganise the astrocytic network around the lesion site and attenuate the astrocyte and microglial cell reactivity. However, ASC do not promote recovery of forelimb motor function.

ACKNOWLEDGEMENTS

I would like to thank friends in the IMB, particularly members of the anatomy and the group and my supervisors; Lev Novikov, Paul Kingham and Mikael Wiberg.

I would especially like to thank Gunnel Folkesson and Gunvor Hellström for their excellent technical assistance and Anita Dreyer-Perkiömäki and Anna-Lena Tallander for their administrative support.

I would like to thank Dag Welin and his wife, Tuva, for suggesting the opportunity to do the research here and subsequently, helping me settle into life in Sweden.

This study was supported by the Swedish Research Council, European Union, Umeå University, County of Västerbotten, Åke Wibergs Stiftelse, and the Clas Groschinskys Minnesfond.

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Department of Integrative Medical Biology
Section for Anatomy
Department of Surgical and Perioperative Scienses
Section for Hand & Plastic Surgery
Umeå University, SE-901 87 Umeå

ISBN: 978-91-7601-007-5