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Transplantation of mesenchymal stem cells and injections of microRNA as therapeutics for nervous system repair

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ABSTRACT

Traumatic injuries to the spinal cord (SCI) and peripheral nerve (PNI) affect several thousand people worldwide every year. At present, there is no effective treatment for SCI and despite continuous improvements in microsurgical reconstructive techniques for PNI, many patients are still left with permanent, devastating neurological dysfunction. This thesis investigates the effects of mesenchymal stem cells (MSC) derived from adipose (ASC) and dental (DSC) tissue and chitosan/microRNA-124 polyplex particles on regeneration after spinal cord and peripheral nerve injury in adult rats. Dental stem cells were obtained from apical papilla, dental pulp, and periodontal ligament. ASC and DSC expressed MSC surface markers (CD73, CD90, CD105 and CD146) and various neurotrophic molecules including BDNF, GDNF, NGF, VEGF-A and angiopoietin-1. Growth factor stimulation of the stem cells resulted in increased secretion of these proteins. Both ASC and DSC supported in vitro neurite outgrowth and in contrast to Schwann cells, ASC did not induce activation of astrocytes. Stimulated ASC also showed an enhanced ability to induce capillary-like tube formation in an in vitro angiogenesis assay. In a peripheral nerve injury model, ASC and DSC were seeded into a fibrin conduit, which was used to bridge a 10 mm rat sciatic nerve gap. After 2 weeks, both ASC and DSC promoted axonal regeneration in the conduit and reduced caspase-3 expression in the dorsal root ganglion (DRG). ASC also enhanced GAP-43 and ATF-3 expression in the spinal cord, reduced c-jun expression in the DRG and increased the vascularity of the implant. After transplantation into injured C3-C4 cervical spinal cord, ASC continued to express neurotrophic factors and laminin and stimulated extensive ingrowth 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. However, ASC did not enhance recovery of forelimb function. In order to reduce activation of microglia/macrophages and the secondary tissue damage after SCI, the role of microRNA-124 was investigated. In vitro

transfection of chitosan/microRNA-124 polyplex particles into rat microglia resulted in the reduction of reactive oxygen species and TNF-α levels and lowered expression of MHC-II. Upon microinjection into uninjured rat spinal cords, particles formed with Cy3-labeled control sequence RNA, were specifically internalized by OX42 positive macrophages and microglia. Alternatively, particles injected in the peritoneum were transported by macrophages to the site of spinal cord injury. Microinjections of chitosan/microRNA-124 particles significantly reduced the number of ED-1 positive macrophages after SCI. In summary, these results show that human MSC produce functional neurotrophic and angiogenic factors, creating a more desirable microenvironment for neural regeneration after spinal cord and peripheral nerve injury. The data also suggests that chitosan/microRNA-124 particles could be potential treatment technique to reduce neuroinflammation.

Keywords: Spinal cord injury, peripheral nerve injury, mesenchymal 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., Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose derived stem cells enhances nerve repair. Stem Cells and Development, 2014, 23, 741-754.

II. **Kolar M.K.**, Itte V.I., Kingham P.J., Novikov L.N., Wiberg M., Kelk P. The neurotrophic effects of different human dental mesenchymal stem cells. Manuscript, submitted to Journal of Dental Research.

III. **Kolar M.K.**, Kingham P.J., Novikova L.N., Wiberg M., Novikov L.N. The therapeutic effects of human adipose derived stem cells in a rat cervical spinal cord injury model. Stem Cells and Development, 2014, 23, 1659-1674.

IV. Louw A.M., **Kolar M.K.**, Novikova L.N., Kingham P.J., Wiberg M,, Kjems J., Novikov L.N. Chitosan polyplex mediated delivery of miRNA-124 reduces activation of microglial cells *in vitro* and in rat models of spinal cord injury. Nanomedicine: Nanotechnology, Biology, and Medicine, 2016, 12, 643–653.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
ALCAR	Acetyl-L-carnitine
ARI	Axon regeneration inhibitors
ASC	Adipose derived stem cell
ATF-3	Activating transcription factor-3
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMSC	Bone marrow mesenchymal stem cell
C3-C4	Cervical spinal cord levels 3-4
CAM	Cell adhesion molecules
CD	Cluster of differentiation
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CFU-F	Fibroblastic colony forming units
CsA	Cyclosporine A
CSPG	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
DAPI	4'-6-Diamidino-2-phenylindole
DePDL	Deciduous teeth stem cells
DFM	Dilute fibrin matrix
DFPC	Dental follicle progenitor cells
DMEM	Dulbecco's modified eagle medium
DPSC	Dental pulp stem cells
DRG	Dorsal root ganglion
DSC	Dental stem cells
ECM	Extracellular matrix
ED-1	Anti-CD68 antibody
EDTA	Ethylene-diamine-tetra acetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether)-tetraacetic acid

EGF	Epidermal growth factor
ESC	Embryonic stem cells
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAP-43	Growth associated protein-43
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
IL-1	Interleukin-1
iPSC	Induced pluripotent stem cells
LIF	Leukaemia inhibitory factor
MAG	Myelin associated glycoprotein
MAI	Myelin associated inhibitors
MEM-α	Minimum Essential Medium, alpha modification
MHC-II	Major histocompatibility complex- class II
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
miRNA	MicroRNA
MSC	Mesenchymal stem cells
NAC	N-acetylcysteine
NCAM	Neural cell adhesion molecules
NG-2	Neural/glial antigen-2
NGC	Nerve guidance conduit
NGF	Nerve growth factor
NgR1	Nogo-66 receptor 1
NPC	Neural progenitor cells
NSC	Neural stem cells
NT-3	Neurotrophin-3
OEC	Olfactory ensheathing cells
OMgp	Oligodendrocyte myelin glycoprotein

OX42	Anti-CD11b/c antibody
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PDL	Poly-D-lysine
PDLSC	Periodontal ligament stem cells
PFA	Paraformaldehyde
PLL	Poly-l-lysine
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
PTRPσ	Protein tyrosine phosphate sigma
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RECA-1	Rat endothelial cell antigen-1
RGM	Repulsive guidance molecule
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SC	Schwann cell
Sema3A	Semaphorin 3A
SCAP	Stem cells from apical papilla
SCI	Spinal cord injury
SHED	Stem cells from human exfoliated deciduous teeth
siRNA	Small interfering RNA
SOX11	SRY-box containing gene 11
SPRR1A	Small proline-repeat protein 1A
TGF- β	Tissue growth factor- beta
TNF- α	Tissue Necrosis Factor-alpha
VEGF	Vascular endothelial growth factor

INTRODUCTION

1. ANATOMY OF THE NERVOUS SYSTEM

The nervous system is conventionally divided into the central and peripheral divisions. The central nervous system (CNS) consists of the brain and spinal cord, whilst the peripheral nervous system (PNS) consists of the cranial nerves, spinal nerves and neurones that constitute the dorsal root ganglia (DRG) and ganglia of the autonomic nervous system. Throughout the nervous system, the number of electrically excitable neurones is vastly outnumbered by the non-electrically excitable glia (supporting cells), which are responsible for creating and maintaining an environment that allows for optimal functioning of the neurones [1].

1.1 The central nervous system (CNS)

The CNS controls the majority of function and is broadly divided into the grey matter (neuronal cell bodies and dendrites) and white matter (myelinated axons), which are kept distinct from each other. Neuronal cell bodies with similar functions that are grouped together are termed nuclei. Such nuclei may be arranged in discrete, non-continuous clusters, such as those found for cranial nerves, or in longitudinal bands, as found within the spinal cord. Predefined tracts of white matter run through the brain and spinal cord. These tracts run through the pyramid of the medulla (Pyramidal tracts) or outside of this (Extrapyramidal tracts). The majority of tracts decussate and thereby supply the contralateral side of the body.

1.1.1 Structure and components of the CNS

The CNS contains a greater range of structures and components than the PNS. For this thesis, the focus is on the broad structures focusing on the spinal cord and spinal nerves.

1.1.1.1 The neurones

Neurones encode information, conduct this along a distance then communicate to other neurones or non-neural cells. Dendrites receive the majority of information at

the synapses via neurotransmitters, transport this to the soma, which passes the message to the next cell through the axons. Axons secrete neurotransmitters across unmyelinated junctions to synapse with dendrites, soma and axons.

1.1.1.2 The glia

The majority of cells with the CNS are the glia, which outnumber neurones by 10-50 times. There are 3 main types within the CNS.

1.1.1.1.1 Astrocytes

The predominant glia, astrocytes provide a network of communication, integration and regulation in the brain. Based on their morphology, astrocytes have been divided into 2 types; fibrous (white matter) and protoplasmic (grey matter) types, although it is not clear if there are any functional differences. Astrocytes are essential in the formation of the Blood-Brain Barrier (BBB), where the astrocytic end feet closely adhere to the basal lamina of the capillaries (see section 1.1.1.2.4).

1.1.1.1.2 Oligodendrocytes

Oligodendrocytes myelinate up to 50 CNS axons each, although in large calibre axons, the ratio may be as low as 1:1. They are arranged longitudinally to the tracts of the axons and are regularly interspersed with astrocytes.

1.1.1.1.3 Microglia

The resident immune cells of the CNS, microglia are thought to be derived from foetal monocytes that migrate into the CNS. They are regularly dispersed throughout the tissue and play a key role in the response to injury.

1.1.1.1.4 The blood-brain barrier

The blood supply to the CNS depends on the anatomical site of interest. However, throughout the CNS, the presence of the Blood-Brain Barrier (BBB) limits the diffusion and transport of various molecules from the blood, making the brain a relatively privileged area. This is achieved by multi-layering of different cell types. The vascular endothelium is surrounded by pericytes, itself covered by the pia mater. The

pia mater is wrapped by the basal lamina, over which the astrocytic end foot is found. Disruption of the BBB occurs in traumatic injuries and contributes to the reaction of the glia following spinal cord injury (SCI).

1.1.2 Pathways of the CNS

1.1.2.1 Ascending pathways of the CNS

Ascending pathways communicate general senses including touch, pressure, vibration, pain, thermal sensation and proprioception from the trunk and limbs through the spinal nerves to the CNS. Primary afferent neurones that receive stimuli for pain, temperature, coarse touch & pressure transport the signal via the DRG, to enter the CNS. They terminate at the dorsal horn of the grey matter of the spinal cord. At this point, they synapse with the secondary, ipsilateral neuronal bodies. These secondary neurones decussate and ascend to the ventral posterior nucleus of the contralateral thalamus as the spinothalamic tract. Here, the secondary neurones synapse with the tertiary neurones. From here tertiary axons pass through the internal capsule to the cerebral cortex where they terminate at the post-central gyrus of the parietal lobe (primary somatosensory cortex).

For primary afferents carrying stimuli for proprioception and fine touch, the primary fibres enter the spinal cord and ascend via the dorsal columns (Fasciculus gracilis & fasciculus cuneatus). The fibres then synapse with secondary fibres in the nucleus gracilis and nucleus cuneatus of the medulla. The secondary fibres decussate within the medulla and ascend in the medial lemniscus to the ventral posterior nucleus of the contralateral thalamus. Here the secondary neurones synapse with tertiary neurones, which pass through the internal capsule terminating at the primary somatosensory cortex.

1.1.2.2 Descending pathways of the CNS

Descending pathways of the CNS provide stimulation to the lower motor neurones to initiate motor signals. They can be broadly divided into the pyramidal and extrapyramidal tracts.

1.1.2.2.1 Pyramidal tracts

Pyramidal tracts denote those that pass through the pyramids of the medulla. These include the corticospinal (CST) and corticobulbar pathways. They are responsible for motor control of the body and face respectively. The corticospinal pathways originate from the primary motor cortex, premotor cortex and supplementary motor areas and to a lesser degree, the somatosensory areas. From the origin in the cortices, the tracts pass through the internal capsule and at the most inferior edge of the medulla, the CST divides into the lateral (75-90%) and anterior CSTs (10-25%). The lateral CST decussates within the pyramid and descend along the lateral funiculus to synapse with the lower motor neurones in the ventral horn of the grey matter of the spinal cord. The anterior CST descends ipsilaterally in the anterior funiculus and decussates in the anterior white commissure to synapse with contralateral neurones.

1.1.2.2.2 Extrapyramidal tracts

Extrapyramidal tracts originate in the brainstem and descend within the spinal cord, to provide motor stimuli for involuntary and automatic responses such as muscle tone, balance, posture and locomotion.

The vestibulospinal and reticulospinal tracts descend ipsilaterally and do not decussate. The vestibulospinal tracts innervate the muscle of posture and balance, whilst the reticulospinal tracts either inhibit movement and decrease tone (lateral reticulospinal tracts) or supplement movement and increase tone (medial reticulospinal tracts).

The rubrospinal (fine movement of the hand) and tectospinal (movement based on vision stimuli) decussate and thereby innervate the contralateral sides.

1.2 The peripheral nervous system (PNS)

The peripheral nervous system provides the pathways for communication between the CNS and the body. This occurs via the cranial nerves (excluding the optic nerve), spinal nerves and a number of autonomic system ganglia that emerge from along the length of the spinal cord.

1.2.1 Structure and components of the PNS

The PNS contains both efferent and afferent fibres. The efferent fibres are the peripheral axonal connections between the neuronal cell bodies found in the CNS grey matter and the effector cells and tissue. Afferent fibres, derived from the dorsal root ganglia (DRG), connect the peripheral receptors to the CNS.

1.2.1.1 The neurones

Neurones found in the PNS are structurally similar to those found in the CNS (see section 1.1.1.1). The function of the PNS is broadly divided into the efferent and afferent systems. The fibres of the PNS vary in diameter and myelination, depending on the functional requirements.

1.2.1.2 Schwann cells

Schwann cells (SCs) are the predominant glia in the PNS. They play a key role in supporting the axons and when greater than 2μm, myelinate the axons. In contrast to oligodendrocytes in the CNS, an individual SC myelinates a single axon. They are essential in the response of the PNS to injury, where they clear the axons distal to the injury zone and realign to form Bands of Büngner to guide the regenerating axons to their targets.

1.2.1.3 The spinal nerves

Peripheral nerve fibres are divided into myelinated or unmyelinated types with differing calibres. PNS fibres are divided into 3 classes (A, B, C; table 1). Class A fibres are further divided into α, β, γ and δ. The largest, fastest conducting fibres are class Aα and class C being the slowest.

Fibre type	Function of fibre	Myelinated/ Unmyelinated	Diameter (μm)	Conduction velocity (m/s)
Aα	Skeletal muscle efferents, afferents of muscle fibres, and tendon organs	Myelinated	20	120
Aβ	Mechanoafferents of skin	Myelinated	6-11	30-60
Aγ	Muscle spindle efferents	Myelinated	1-6	2-30
Aδ	Skin afferent (Temp & fast pain)	Myelinated		
B	Visceral afferents	Myelinated	3	3-15
C	Postganglionic efferent fibres	Unmyelinated	0.5-1-5	0.25-1.5

Table 1: *Classification of PNS nerve fibres based on fibre types*

The connective tissue of the peripheral nerves consists of 3 layers; epineurium, perineurium and endoneurium.

1.2.1.3.1 The epineurium

The epineurium is the outermost covering of the nerves. It is a condensation of the loose areolar tissue and in generally thicker in those nerves that have a higher number of fasciculi. Consisting of collagen (types I and III), fibroblasts and fat, the epineurium provides cushioning from the surrounding structures. Within this layer runs the vasa nervosum and lymphatics.

1.2.1.3.2 The perineurium

The middle of the 3 layers, the perineurium runs from the CNS transition zone to the capsule of the muscle fibres. It consists of collagen and fibroblasts and covers individual fascicles. The perineurium plays a key role in the blood nerve barrier and maintaining the homeostatic environment.

1.2.1.3.3 The endoneurium

The endoneurium is the intrafascicular connective tissue and consists of type III collagen. It runs longitudinal to the axonal axis and condenses around the Schwann cell-axon units and endoneurial vessels. Within this layer lie the Schwann cells, fibroblasts, macrophages and mast cells.

1.2.1.4 The blood supply to the PNS

The blood supply to the PNS axons occurs via 2 independent systems; the extrinsic regional nutritive and epineural vessels and intrinsic microvessels systems running within the endoneurium. There are a significant number of anastomoses that allow for greater resistance to ischaemia in the PNS system.

At the level of the endoneurium, in a similar configuration to the BBB in the CNS (see section 1.1.1.2.4), the blood-nerve barrier, at the level of the endoneurial capillaries provides a degree of protection to the axons, although this is less so at the DRG.

1.2.1.5 Peripheral nerve pathways

1.2.1.5.1 The efferent system

The efferent fibres run from the centrally placed neurones within the grey matter away from the CNS. Such tracts may run directly from distantly placed neurones or via interneurones communicating via more locally found neurones. Efferent fibres exit the spinal cord as the ventral root, via the ventral spinal foramina. The axons synapse with the target organ (muscle/glands) to initiate the desired response.

1.2.1.1.2 The afferent system

The neuronal cell bodies of the afferent system are found in the DRG. The afferent fibres run from the end organ towards the CNS and communicate with it within the dorsal horn of the grey matter of the spinal cord, via interneurones locally or more distantly placed. Afferent fibres provide feedback for a number of different stimuli including mechanoreceptor, chemoreceptor, photoreceptors and thermoreceptors.

2. 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 [2]. 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 [3], with the primary cause of injury being motor vehicle collisions and falls, respectively [4]. The sex ratio varies between 2-6.7:1 (Male:Female; M:F) [3]. 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 [5].

Peripheral nerve injuries (PNI) occur more frequently, with an annual incidence of 13.9 per 100,000 [6]. Again such injuries predominantly affect young males (aged 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 individuals affected with PNI are of working age (20-64 years) [6]. In digital nerve injuries, Thorsen et al. found that the direct costs of treatment, including hospital stay, operation, outpatient visits and 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 [7]. 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.

2.1 Pathophysiology of spinal cord and peripheral nerve injury

2.1.1 Spinal cord injury (SCI)

The effects of SCI occur as a result of two phases. Primary damage occurs as a result of direct trauma (sharp injury/compression/contusion) [8]. The mechanical injury leads to secondary injury involving biological reactions, beginning minutes after the injury and continuing up to years later. Although the processes are not distinct, secondary damage can be divided into three phases; acute, sub-acute and chronic.

2.1.1.1 Acute and sub-acute SCI

The vascular effects of SCI include haemorrhage, vasospasm, thrombosis, and loss of autoregulation, breakdown of the blood - brain barrier and infiltration of inflammatory cells. The result is oedema, necrosis and ischaemia [9,10].

Production of free radicals causes oxidative death in spinal cord neurones and reduces the spinal cord blood flow leading to oedema and an inflammatory response [11-13]. Free radicals react with the polyunsaturated fatty acid of the cell membrane leading to peroxidation and disruption of the normal phospholipid architecture of both the plasma- 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], resulting in loss of neuronal function and this ultimately leads to tissue dissolution [15].

Disruption of K^+, Na^+, Ca^{2+} ionic balance can affect depolarisation of cell membranes, ATPase failure and increase of intracellular Ca^{2+} [16] and thus, potentiate cell death. 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]. There is strong morphological and biochemical evidence for the occurrence of apoptosis in neurones, 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].

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 leucocytes 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.

2.1.1.2 Chronic SCI

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].

2.1.1.3 Hurdles following SCI

Neurones in the CNS are capable of long distance growth but this rarely happens. The greatest impediment to this is the glial scar.

2.1.1.3 .1 Inflammatory cells and reactive astrocytes

Following SCI, disruption of the BBB results in leakage of blood and serum components. With it, a number of factors are brought to the injury zone including interleukin-1 (IL-1), transforming growth factor- β (TGF- β) and fibrinogen [28]. This is closely followed by extravasation of leucocytes, which coalesce at the core of the

injury scar. As a result of polarisation, ED-1 positive peripheral macrophages and to a lesser degree [29], CNS microglia migrate to the injury zone [30]. The influx of microglia/macrophages result in production of matrix metalloproteinase (MMP), which enhance vascular permeability and thus, further recruitment of macrophages [31]. Macrophages are essential in clearance of the debris, but also play an important role in SCI secondary damage via the inflammatory process. The role of the macrophage/microglia population following SCI is dependent on the pro-inflammatory M1 or anti-inflammatory/immune-regulatory M2 polarisation. M1 are predominant early following demyelination and contribute to further inflammatory recruitment. The switch to a M2-dominant macrophage profile is required for the regenerative phase to begin [32].

Migration of the microglia/macrophage complex drives astrocytes away from the epicentre of injury [33]. Hypertrophy of the activated astrocytes at the lesion penumbra leads to increased glial fibrillary acidic protein (GFAP), vimentin and nestin production. A mesh-like structure forms and thus creates the major component of the glial scar, preventing regeneration of long descending and ascending axons. Chondroitin sulphate proteoglycans (CSPG) produced by reactive astrocytes contribute to the biochemical component to the glial scar. CSPG levels are elevated within 24 hours post injury and can persist for months. The primary role of the scar is prevention of secondary axonal damage as a result of macrophage migration [28].

Peripheral fibroblasts migrate to the lesion core and separate themselves from the astrocytes through bi-directional ephrin B2 signalling. Fibroblasts also release fibronectin, collagen and laminin; a range of molecules to repulse axonal regeneration and potentiate astrocytic reactivity at the scar [28].

SCI leads to migration of neural/glial antigen-2 (NG-2) positive oligodendrocytes. These cells support the dystrophic axonal ends through synaptic connections and provision of substrates [34]. However, oligodendrocytes are also responsible for the production of CNS myelin which is one of the major chemical barriers to CNS axonal regeneration [35].

2.1.1.3.2 Inhibitors of axonal regeneration

Broadly, there are two major categories of chemical axonal regeneration inhibitors following SCI.

CSPGs are the predominant category of extracellular inhibitors, found within the perineurial nets. They include neurocan, aggrecan, brevican, phosphocan, versican and NG-2. CSPG receptors include protein tyrosine phosphate sigma (PTRPσ) and NgR1 Removal of CSPGs has been shown to have a significant effect on regeneration [36], and thus is an increasingly focused target in SCI.

Myelin associated inhibitors (MAI) including Nogo-A, myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), ephrins-B3 & semaphorin 4D [37-41] are components of CNS myelin expressed by oligodendrocytes. Nogo-A, MAG and OMgp limit axonal regeneration through the neuronal Nogo-66 receptor 1 (NgR1) [42]. Inhibition of Nogo-A has been shown to significantly improve regeneration of CST and raphespinal tracts [43]. In contrast, the results following inhibition of MAG and OMgp have been less impressive. An alternative option is blocking or competitive antagonism of the NgR1 receptor which results in CST and raphespinal regeneration, thus emphasising its importance in inhibition of CNS regeneration [35].

Other axon regeneration inhibitors (ARI) not found in myelin or the glial scar include semaphorin 3A (sema3A) and repulsive guidance molecules (RGM). Previous studies have shown that inhibition of these molecules promotes functional recovery following SCI [44,45].

2.2 Peripheral nerve injury (PNI)

Classification of peripheral nerve injury is based on the work of Seddon and Sunderland [46,47] 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 nerve structure (neurotmesis) and affects both primary sensory and motor neurone cell bodies residing in the DRG and spinal cord, respectively.

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 [48].

Using transections as the example for responses to PNI, the process can be subdivided into those of the cell body, injury zone and target organ.

2.2.1 Response at the cell body

The earliest indication of axonal damage to the soma is from the antidromic passage of electrical activity from the injury zone. This results in the opening of calcium channels and initiation of Jun-kinase cascades that influence transcription. It is generally accepted that the most important determinant for neuronal survival is the availability of target-derived neurotrophic support [49,50]. The intracellular balance between *de novo* gene expression and production of neurotrophic factors determines whether the neurones survive and attempt regeneration or undergo apoptosis [51-53]. Interestingly, primary sensory DRG neurones are 40% more likely to undergo apoptosis in comparison to spinal motor neurones following injury [54].

2.2.2 Responses at the injury site

Within a few hours, the distal stump of the injured nerve undergoes anterograde Wallerian degeneration, both the axon and the myelin in the distal stump degenerate and macrophages migrate to the site of injury and contribute to debris clearance [9,18,19]. In the first 24 h, SCs proliferate and switch from a myelinating to a regenerative phenotype and exhibit up-regulation of several molecules that assist the

parallel degenerative and regenerative processes [18,19]. When the debris has been cleared by the combined action of SCs and macrophages, SCs align forming cellular columns called bands of Büngner. This forms a permissive environment rich in trophic factors, enabling guided axonal regeneration [22]. At the same time, SCs depend on the contact with regenerating axons to receive trophic molecules for survival (e.g. neuregulins).

In the first 24 h following injury denervated SCs proliferate and switch to the regenerative phenotype. This results in altered expression of a range of molecules to assist in the degeneration and repair process. Down regulation occurs of structural proteins such as protein zero, myelin basic protein and myelin-associated glycoprotein. Concurrently, up regulation occurs of cell adhesion molecules (CAM; L1, neural CAM (NCAM), and glial fibrillary acidic protein (GFAP)), and growth factors (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), fibroblast growth factor -2 (FGF-2) and neurotrophin 3 (NT-3)) [55,56].

Within hours of injury, Wallerian degeneration occurs, involving physical fragmentation of axons and myelin distal to the site 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.

The distal axonal and myelin debris is disintegrated by SCs and the remains phagocytosed, alongside macrophages, from the site of injury in a process requiring between 1 week to several months. At later stages, SCs align themselves to form the 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 [48].

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 SCs within an endoneurial sheath, remain.

2.2.3 Responses at the target organ

In order to successfully reinnervate the target organ, a number of challenges exist for the axons. Misdirection towards the wrong target reduces functional outcome despite significant axonal regeneration across the injury zone. Attenuation of the growth cones prevents misdirected regeneration, minimising the number of axons that do not reach the correct target or lose support of their endoneurial tubes [57]. A lack of neuronal contact in the distal stump leads to chronically denervated SCs, which down regulate growth factors thus entering a dormant state, unable to support axonal regeneration [58]. Similarly, once the denervated target organ is exhausted of trophic factors, the muscle fibres atrophy and satellite cells undergo apoptosis [59].

2.2.4 Hurdles following PNI

PNS neurones produce a greater number of regenerative associated genes including c-jun (which promotes growth state and more efficient axonal growth), activating transcription factor-3 (ATF-3; promotion of neurite outgrowth), SRY-box containing gene 11 (Sox11; efficient nerve growth), small proline-repeat protein 1A (SPRR1A; promotion of neurite outgrowth), growth-associated protein-43 (GAP-43) and CAP-23 [35]. Despite the comparatively greater regenerative potential of PNS axons, clinical outcomes are poor (see section 2). Limitations to this include neuronal survival, distance between nerve stumps and adequate stimulation of the target organ until reinnervation occurs.

Following PNI, it has been demonstrated that sensory neurones are more likely to undergo apoptosis than affected motor neurones [54]. In order to promote survival of the neuronal bodies, pharmacological therapies such as neurotrophic factors and antioxidants including N-acetylcysteine (NAC) and acetyl-L-carnitine (ALCAR) have

been shown to be safe systemically and provide complete neuroprotection [60]. Other alternatives include SCs and stem cells, through provision of neurotrophic support [61].

At the injury zone, distances of greater than 5 cm in human nerves pose a significant challenge, as tension-free primary is not feasible. The gold standard in such cases is the autologous nerve graft because denervated SCs up-regulate expression of various neurotrophic molecules [62]. As a natural, non-immunogenic, ready-to-use graft, this method has clear advantages, but functional outcome remains poor with only 50% of patients achieving successful outcomes [63,64]. The use of a sensory nerve graft can be limiting when used for pure motor or mixed nerve injuries [65,66] due to the morphometric mismatches in environments, axonal alignment, distribution and size [65,67]; motor axons typically range between 3 and 20μm and sensory nerves between 0.2 and 15μm [68]. More recently, the differences between motor and sensory SC modalities has been noted [69] and if in the wrong environment, the regenerative ability of the graft is impacted. Also, for the patient, there is additional donor site morbidity, scarring, sensory loss and possible neuroma formation [70,71]. To search for an alternative solution to nerve grafting, much research has been performed concerning the construction of artificial bioengineered conduits. A significant number of considerations must be taken into account for these to replicate the graft (see section 5.3), with varying degrees of success achieved [72].

The denervated target organ must be considered within the regenerative process. Although sensory endings are can be successfully renervated after years, muscle atrophy is more difficult to manage [61]. If denervated for long periods, atrophy and fibrosis occurs, making successful use of the muscle impossible. In such cases muscle transfers may be attempted. At an experimental level, local provision of growth factor as individual factors (vascular endothelial growth factor, VEGF; nerve growth factor, NGF; ciliary neurotrophic factor, CNTF; glial cell derived neurotrophic factor, GDNF; insulin like growth factor, IGF) reduce muscle atrophy [73]. In addition, intramuscular injection of stem cells has improved functional outcomes by preserving muscle [74,75].

3. REGENERATION FOLLOWING INJURY

3.1 Regeneration after SCI

There is very limited regeneration in the trauma zone. Recovery following SCI has been reported to primarily occur due to plasticity and sprouting in supraspinal and propriospinal networks. This was first suggested by the observation that paralysed cats with complete spinal cord transection could be trained to walk on a treadmill [76] 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 [77] 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 [78], of spinal interneurones [79], adaptations of the motor neurones caudal to the injury [80], and by functional redundancy [81].

More recently, axonal regeneration in SCI has been shown to be possible when the inhibitory environment of the glial scar is modified [82]. CNS neurones have demonstrated the capacity to regenerate for long distances, when directed through the more growth permissive environment of the peripheral nerve graft, but this regeneration is then halted upon contact with the reactive CNS environ. Regeneration is limited by the lower levels of growth promoters expressed by neurones in the CNS in addition to the potent growth inhibitors expressed (see section 4) [83,84].

3.2 Regeneration after PNI

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 [85], as tension to re-oppose the cut edges leads to ischaemia and thereby reduces the ability to regenerate. 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 [86] and only about 50% of all injured sensory and motor neurones are capable to send their axons across the injury site into the distal nerve stump [60]

The process of regeneration may last up to 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 up to the end organ and finally the end organ itself [48]. Changes occur to allow for axonal regeneration beginning from the neuronal body, with multiple axons sprouting 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.

4. STRATEGIES TO PROMOTE REGENERATION AFTER SCI AND PNI

4.1 Cell transplantation

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 [87,88]. One method of targeting these different needs may be through transplantation of cells. A number of different cell populations have been used for this purpose, ranging from various types of glial cells to stem cells [89,90].

4.1.1 Transplantation of glial cells

Glial cells that have been studied include olfactory ensheathing cells (OEC) and SCs. The rationale for use of OEC stems from phenotypic features of both astrocytes and SCs. In addition, they have been found to secrete a range of growth factors. However, outcomes vary. Use of SCs in nerve injury is based on the growth permissive environment in the PNS and the ability to encourage CNS axons to regenerate long distances. However, the SC-astrocyte interface is known to cause increased reactivity of the astrocytes and thus hinder re-entry of CNS axons [90]. In contrast, SCs are ideal to aid the regenerative process following PNI as they are the endogenous resident glia. However, harvesting autologous SC requires the sacrifice of a healthy nerve in combination with prolonged culture times, thereby limiting their potential uses.

4.1.2 Transplantation of stem cells

The ideal stem cell 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 [91]. Both embryonic and adult tissue-derived stem cells have been shown to promote nervous system regeneration [91], but widespread use of embryonic stem cells (ESC) is unlikely given the ethical issues concerning their derivation. Induced pluripotent stem cells (iPSC) may offer an alternative to ESC but it has been shown that they are less likely to undergo neural differentiation than ESC [92]. However, when differentiated into each neural lineage, the iPSC can integrate and improve clinical outcomes without

evoking tumourigenicity [93]. Neural stem cells (NSC) have shown similarly positive outcomes with good clinical outcomes, including when tested in human subjects [90].

From a clinical standpoint, both Schwann cells and adult mesenchymal stem cells represent important sources of cells for neural transplantation due to their availability and relatively simple culture protocols. Mesenchymal stem cells (MSC) can be obtained from any tissue of mesenchymal origin, including bone marrow (BMSC), adipose tissue (ASC), dental tissue (DSC), and perinatal sources (umbilical cord blood cells, Wharton's Jelly, and placental MSC) [94]. They are characterised based on the ability to form fibroblastic colony forming units (CFU-C), plastic adherence and the ability to differentiate into osteoblasts, adipocytes and chondroblasts. Transplanted MSC have demonstrated beneficial functional effects [95-97] that could be attributed to production of various neurotrophic factors [98-101], immunomodulation with attenuation of astrocytic and microglia/macrophage responses [102-104], modification of extracellular matrix molecules [105], and degradation of inhibitory CSPGs [106,107]. The most extensively studied are BMSC but interest has grown in ASC and more recently DSC. Recent studies have demonstrated the ability of MSC to support axonal regeneration [91,108,109], and this effect has been primarily attributed to the environmental support provided by the stem cells during regeneration. They have also demonstrated the ability to protect neurones, promote angiogenesis and provide neurotrophic factors for regenerating axons and protect target organs [110,111].

4.2 MicroRNA for SCI

MicroRNAs (miRNAs) belong to a family of small non–protein coding RNAs that regulate expression of multiple target genes and are involved in many fundamental biological processes, including embryonic development, cell proliferation, differentiation and apoptosis. Functionally, miRNAs promote degradation of messenger RNA (mRNA) or prevent translation of the target genes. Therefore, miRNAs can be considered to be endogenous mediators of RNA interference [112].

Expression pattern	**miRNA detected at time points**				
	4 hours	1 day	4 days	7 days	>7 days
Upregulated	miR-1, miR-15b, miR-20a, miR-20b-5p, miR-21, miR-30a, miR-31, miR-92a, miR-92b, miR-93, miR-98, miR-106b, miR-145, miR-146b, miR-152, miR-199a-3p, miR-203, miR-206, miR-214, miR-221, miR-223, miR-290, miR-333, miR-374, miR-378, miR-672, miR-674-5p, miR-872	miR-17, miR-146a, miR-124, miR-486		miR-486	
Downregulated	miR-30b-5p, miR-30c, miR-30d, miR-34a, miR-129, miR-137, miR-138, miR-219-2-3p, miR-219-5p, miR-323, miR-325-3p, miR-338, miR-379, miR-384-5p, miR-495, miR-543	miR-146a, miR-708, miR-125b-3p, miR-126, miR-let-7b	miR-129-1, miR-129-2	miR-129-3p, miR-342	miR-199a-3p

Table 2: Expression profiles of various miRNA following SCI

In response to SCI, miRNA expression profiles may be up-regulated, down regulated or show bidirectional changes. Previous studies have shown that following SCI, expression of almost 300 miRNAs altered in response, although significance was found in only 97 miRNAs. Of these, 60 were found to have upregulated greater than 500 fold whilst the remaining were less than 500 fold [113]. In general, it was noted that miRNAs targeting anti-inflammatory and anti-apoptotic mRNA increased, while miRNAs targeting pro-inflammatory and pro-apoptotic mRNA decreased (Table 2). The number of down regulated miRNAs increased from the time of injury while the number of upregulated miRNAs remained constant [113].

miRNA-124 is expressed by CNS neurones and plays an important role in differentiation of neural progenitors to mature neurones [114]. In addition, miRNA-124 is expressed by resting resident microglia. Following SCI, microglia down regulate miRNA-124. However, increases in its expression lead to decreases in activated microglia and deactivation of peripheral macrophages [115].

One method of delivering miRNA is within inert chitosan particles. These have successfully transported small interfering RNA (siRNA) *per os* to the target organ [116]. A number of different methods of delivery have been trialled including *ex vivo* transplantation, intravenous injections and topical applications [117].

4.3 Biosynthetic conduits for peripheral nerve repair

Clinically, the limited access to autologous donor material and the problems with allograft rejection has prompted a search for immunologically inert bridging biomaterials. 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 [85].

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 [118].

A nerve conduit has a number of criteria to fulfil in that it should; (i) direct axon sprouting from the proximal to distal stumps; (ii) maintain adequate mechanical support for the regenerating nerve fibres; (iii) provide a conduit channel for the diffusion of neurotrophic factors secreted by the damaged nerve stump and a conduit wall for the exchange of nutrients and waste products; (iv) obviate the infiltration of fibrous scar tissue which will hinder axon regeneration; and (v) create an optimal microenvironment for nerve regeneration through the accumulation and release of exogenous and endogenous biochemical effects [72].

A major development in the construction of biosynthetic nerve 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 [119,120]. This has meant a threefold approach to construction of a conduit through optimisation of (i) the conduit's body, (ii) the intraluminal structure and (iii) the intraluminal contents (e.g. by addition of growth factors or regenerative cells.

There are a small number of commercially available nerve conduits manufactured to bridge the 'nerve gap' in PNI patients (AxoGuard® Nerve Connector; http://www.axogeninc.com; NeuraGen® Nerve Guide, http://www.integralife.com; Stryker NeuroMatrix, https://www.stryker.com). They are made from different biodegradable materials in the form of an empty tube and those currently approved are clinically effective for only small gaps. Their underperformance is most likely attributed to the lack of both a supportive matrix and a growth-promoting cellular environment.

AIMS OF THE STUDY

The specific aims of the study were as follows:

- To evaluate the neurotrophic and angiogenic properties of human adipose derived and dental mesenchymal stem cells and their effects on the retrograde reaction in sensory and motor neurones and axonal regeneration in a peripheral nerve injury model in adult rats (Papers I & II).

- 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 III).

- To study the effects of chitosan/microRNA-124 microparticles on activation of microglial cells *in vitro* and *in vivo* following traumatic spinal cord injury (Paper IV).

MATERIALS AND METHODS

5. CELL CULTURE

5.1 Culture of human adipose derived stem cells (ASC)

Human adipose tissue samples were obtained from abdominal fat from a total of 5 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 [121]. The final stromal fraction pellet containing stem cells was resuspended in growth medium (Minimum Essential Medium Alpha modification; 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 (HBSS) 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.

5.2 Culture of human dental MSC (DSC)

All procedures were approved by the local ethics committee for Clinical Research in Umeå University (No. 03-425). Impacted third molars with 30-50% root formations were surgically removed (n=7; age range 15-25 years) at the Maxillofacial Surgery Section at University Hospital, Umeå. Human stem cells from apical papilla (SCAP), dental pulp stem cells (DPSC), and periodontal ligament stem cells (PDLSC) were isolated from the teeth by mincing followed by digestion in 3mg/ml collagenase type I (Worthington Biochemicals Corp.) and 4 mg/ml dispase II (Roche Diagnostic/Boehringer Mannheim Corp.) for 60 minutes at 37˚C and 5% CO_2. Single cell suspensions of SCAP, DPSC and PDLSC were obtained by passing the cells through a 70 μm strainer (Falcon, BD Labware). Cells were seeded at 10000 cells/25cm^2 flask (Costar), and cultured in MEM-α supplemented with 15% (v/v) FCS,

100 mM L-ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), 2 mM L-glutamine (Biosource/Invitrogen) and 1% (v/v) penicillin/streptomycin solution (PAA) at 37°C in 5% CO_2. On reaching ~90% confluence, cells were passaged to new 75cm^2 culture flasks using trypsin/EDTA solution (Invitrogen) and plated at a density of 5,000 cells/cm^2. DSC were successfully expanded from two of the seven donors through to passage 4 (P4), as previously described [122,123]. Cells between p1-4 were used in *in vitro* and *in vivo* experiments; comparisons were made on cells at matching passage numbers.

5.3 Stem cell characterisation

The multipotency of the stem cell populations were demonstrated by differentiation into osteogenic, and adipogenic lineages as described previously [124]. ASC were characterised by immunocytochemistry for MSC markers (see section 8.6) Flow cytometry was used to characterise the DSCs at early passage (p1-2). Cells were collected and tested for positive MSC-associated surface markers (CD73, CD90, CD105, and CD146) and negative markers (CD11b, CD19, CD34, CD 45 and HLA-DR), to define the cells as MSCs [125] according to the manufacturer's protocol (BD Bioscience). All antibodies used for FACS analysis were PE-conjugated. Optimal concentrations of antibodies were calculated and 5000 cells for each analysis were chosen. As negative control, a corresponding isotype control was used for each sample (mouse IgG1, κ). Data was acquired using FACS^{calibur} (BD Bioscience).

5.4 Stimulation of stem cell populations with growth factors

For Papers I & II, ASC and DSC were treated to stimulate an enhanced neurotrophic and angiogenic phenotype, as previously described for rat cells [124]. 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; Millipore), 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.

5.5 Culture of DRG neurones and neurite outgrowth assay

Cultures of DRG neurones were prepared from adult rats (n=6) as described previously [126,127]. The DRG neurones 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 neurones 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 and DSC from different patients.

5.6 Culture of cortical astrocytes

For Paper I, primary cultures of astrocytes were prepared from cerebral cortices obtained from 5-day-old rats as described previously [128]. 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.

5.7 Culture of Schwann cells

For Papers II and III, primary cultures of Schwann cells were prepared from the sciatic nerves of adult rats as described previously [129]. In brief, sciatic nerves were dissected into DMEM with 10% (v/v) FCS, cut into small pieces and incubated at 37°C and 5% CO2 for 2 weeks. After enzyme digestion and trituration, the cells were plated onto 25cm^2 poly-D-lysine (PDL) coated tissue culture flasks with Schwann cell growth medium, supplemented with 10μM forskolin (SigmaAldrich) and 100ng/ml 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.

5.8 Confrontation assay of astrocytes with ASC and Schwann cells

For Paper III, 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 50 μl 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 neurones 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) paraformaldehyde (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.

5.9 *In vitro* angiogenesis assay

For papers I & II, 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 the factors used to stimulate ASC interfered with the assay, the ASC were first stimulated for 24 h, then washed four times with HBSS to remove stimulating factors, and regular growth medium re-applied 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.

6. 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.

6.1 Peripheral nerve injury model

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™ 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[108]. After glue polymerization, the rods and silicone mould were removed, and fibrin glue

conduits were loaded with fibrin matrix with or without cells (Table 3). Fibrin matrix was produced by modifying the fibrin glue as previously described [130]. Stem cells (unstimulated or stimulated) 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.

6.2 Spinal cord injury model

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 [131].

Conduit contents	**Cell type**	**Cell number**
Paper I groups		
DFM	-	-
DFM + ASC	Unstimulated human ASC	2 x 10^6/20 µL
DFM + sASC	Stimulated human ASC	2 x 10^6/20 µL
Paper II groups		
DFM	-	-
DFM + SC	Rat Schwann Cells	2 x 10^6/20 µL
DFM + SCAP	Unstimulated human stem cells from apical papilla	2 x 10^6/20 µL
DFM + DPSC	Unstimulated human dental pulp stem cells	2 x 10^6/20 µL
DFM + PDLSC	Unstimulated human periodontal ligament stem cells	2 x 10^6/20 µL

Table 3: Conduit contents for PNI models. DFM- dilute fibrin matrix, ASC- Adipose derived stem cells, sASC- stimulated adipose derived stem cells, SC- Schwann cells, SCAP- Stem cells from apical papilla, DPSC- Dental pulp stem cells, PDLSC- Periodontal ligament stem cells.

6.3 Injection of polyplex particles following SCI

Chitosan polyplex particles (provided by Kjems et al.) were created with either Cy3 label, miR control or miR-124. For injections of particles into the normal spinal cord (paper IV), cervical C3-C4 laminectomy was performed and rats were mounted in a stereotaxic frame (Stoelting's Lab Standard Stereotaxic Instrument, Stoelting Co., USA). A glass micropipette (outer tip diameter 70 µm) was attached to a 5 µl Hamilton syringe, filled with 35 ng µl 262 -1 miRNA particles suspended in acetate buffer pH 5.5, and 1 µl of particles was slowly (10 minutes) pressure-injected into the lateral

funiculus (depths 1.0 mm) in 3 sites along rostro-caudal axis of the C3-C4 spinal segments at approximately 1 mm from each other. The micropipette was left in place for additional 2–3 min. Dura mater was covered with Spongostan®, muscles and skin were closed in layers.

For injections of particles in animals with spinal cord injury, the C3 spinal cord segment was exposed and transected on the left side with fine scissors under an operating microscope. The lesion included the lateral funiculus as well as the adjacent grey matter. Particles were pressure-injected into the lateral funiculus (depths 1.0 mm) at approximately 1 mm rostral and 1 mm caudal to the lesion site as described above. For intraperitoneal injections, 1 ml solution of particle labelled with Cy-3 was used. In a separate group of animals, the trauma zone was expanded in the rostro-caudal direction by gentle aspiration to create a 1–2mm long cavity in the C3 spinal segment. A small piece of Spongostan® was soaked in 1 µl of 35 ng µl−1 miRNA particles suspended in acetate buffer pH 5.5 and implanted into the cavity. The injury site was covered with fibrin glue (Tisseel® Baxter SA, Switzerland) and muscles and skin were closed in layers.

7. CELL AND TISSUE PROCESSING AND ANALYSIS

7.1 Tissue harvest

Cultured cells were fixed with 4% (w/v) PFA 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 following SCI, the rats were transcardially perfused with Tyrode's solution followed by 4% (w/v) PFA in 0.1M phosphate buffer (pH 7.4) and then either separate cervical spinal cord segments were removed (C2, C3-C5 & C6 at 2 and 8 weeks; paper III) or *en bloc* (C2-C5 at 3h, 24h, 2, 3 & 7 days; paper IV), 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.

7.2 RT-PCR

RT-PCR was performed to qualitatively measure mRNA expression levels. Total RNA was isolated from cells 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 I, Figure 2), 10ng RNA extracted from tissue and transplanted cells (See Paper I, Figure 3) or 1 ng RNA was isolated from human ASC and DSC (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 I, 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.

7.3 qRT-PCR

For Paper I, 10ng 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 III, 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 III, 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.

7.4 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.

7.5 Enzyme-linked immunosorbant assay (ELISA)

For papers II & III, stem cells (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 incubation.

7.6 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 papers I, II and III, the following primary antibodies were used: mouse anti-CD14 (1:500; Millipore AB), mouse anti-CD19 (1:500; Millipore AB), mouse anti-CD54 (1:20; Millipore AB), mouse anti-CD90 (1:200; Millipore AB), goat anti-CD105 (1:20; R&D System), mouse anti-CD146 (1:200; Millipore AB) mouse anti-human cell adhesion molecule (HCAM or CD44; 1:200; Millipore AB) mouse anti-fibronectin (1:500; Millipore AB), mouse anti-laminin (1:500; Millipore AB). In papers I and II, antibodies used were: 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 III 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.

7.7 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.

7.8 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).

7.9 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.

7.10 Behavioural testing of spinal cord injured animals

For Paper III, 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 [132,133]. 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.

7.11 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 $^{***}P < 0.0$

RESULTS

8. HUMAN STEM CELLS IN CULTURE

8.1 Stem cell characterisation

Cultured ASC and DSC 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 III, 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 I, 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).

Adherent DSC cultures from early passages were analysed using flow cytometry. All three populations showed high expression of mesenchymal stem cell markers (CD73, CD90, CD105, CD146) and negligible expression of the negative control markers of CD11b, CD45, HLA-DR, CD19 and CD 34. Each type of DSC efficiently differentiated along the osteogenic lineage shown by Alizarin red positive calcium staining.

8.2 Stimulation of stem cells 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, *NGF, BDNF, GDNF* but down-regulated the expression of *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 II, Fig. 2D).

Semi-quantitative RT-PCR was used to assess the neurotrophic and angiogenic factor profiles of each DSC population from both patients. For comparison, a housekeeping gene (*GAPDH*) and an embryonic stem cell marker gene (*nanog*) were used. The gene expression of *nanog*, *BDNF, GDNF*, *angiopoietin-1* and *VEGF-A* was highest in SCAP followed by DPSC and PDLSC in both patients (See paper II, Figure 2). There was some variation in *NT-3* expression levels and the DPSC tended to have the lowest levels of expression. *NGF* expression was similar between DSC in patient I but PDLSC were found to have highest expression in patient II.

The levels of growth factors secreted from DSC were also determined by ELISA. In addition to baseline release, the levels of growth factors were measured after *in vitro* stimulation using a protocol which was shown to boost neurotrophic activity of adipose derived stem cells [134]. Stimulation of DSC led to a significant increase in the production of BDNF in SCAP and DPSC but not in PDLSC (See paper II, Figure 3A). In contrast, stimulation of DSC led towards a trend of decreased production of NGF (See paper II, Figure 3B). In addition, no changes in the production of NT-3 or GDNF were observed in any of the stimulated DSC (See paper II, Figure 3C & D). On the other hand, all three types of DSC significantly increased VEGF-A production following the stimulation protocol (See paper II, Figure 3E), while the same treatment decreased production of angiopoietin-1 (See paper II, Figure 3F).

8.3 Effects of ASC and DSC on *in vitro* neurite outgrowth

In vitro assays for neurite outgrowth were used to determine whether the increased levels of the growth factors had some functional consequences in the presence of ASC. Rat DRG neurones were seeded onto coverslips and after 48 h, they were immunostained with β-III-tubulin antibody to measure neurites. Neurones seeded in medium conditioned by ASC or stimulated ASC extended a number of long neurites that were absent in the DRG neurone 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 (μm) were significantly higher in neurones treated with stimulated ASC conditioned media compared with ASC conditioned media (See Paper I, Fig. 3B,C).

To examine the *in vitro* biological activity of the conditioned media from DSC another neurite outgrowth assay was used. Neurally-differentiated SH-SY5Y cells were incubated for 24 hours in media taken from the unstimulated or stimulated DSCs (See paper II, Figure 4A). Quantification of the neurite outgrowth showed that unstimulated and stimulated DSC increased both the percentage of cells producing neurites (See paper II, Figure 4B) and the total neurite outgrowth (See paper II, Figure 4C), when compared with the respective medium-only controls. However, the length of the longest neurite per neurone was not significantly increased in the presence of cell-conditioned media (See paper II, Figure 4D). Although there were no statistically significant differences between corresponding unstimulated and stimulated DSC groups, SCAP tended to perform better than DPSC and PDLSC in each measured parameter**.**

8.4 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 potentiation 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).

8.5 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 III, Fig. 3A,B). To investigate how the interaction of astrocytes with Schwann cells or ASC could influence axonal growth *in vitro*, 5000 adult DRG neurones were applied along the border between the interacting cells for 24 hours. In the co-cultures of astrocytes and Schwann cells, most neurones that landed on the interface between two cell territories grew their axons in close association with Schwann cells (See Paper III, 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 neurones had no preferential growth and axons were found on both cell types regardless of their initial attachment (See Paper III, 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 III, Fig.3C). Thus, there were increased transcript levels for *GFAP*, the growth inhibitory CSPGs, *neurocan* and *aggrecan*, extracellular matrix molecule *tenascin C*, pro-inflammatory cytokine *TGF-β* and adhesion molecule *β1integrin* (See Paper III, Fig. 3C).

9. TRANSPLANTATION OF HUMAN STEM CELLS AFTER PNI

9.1 Effects of stem cells on regeneration 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).

Unstimulated DSC were seeded in fibrin conduits and tested for their effects on nerve regeneration *in vivo*. At 2 weeks after sciatic nerve injury, immunohistochemistry demonstrated the greatest distance of regeneration occurred in rats treated with SCAP-seeded conduits, which was greater than in animals treated with rat Schwann cells (See paper II, Figure 5A,C). Although less than the SCAP and rat SC groups, the increase in the distance of regeneration in conduits seeded with DPSC and PDLSC was statistically significant (See paper II, Figure 5A & C). Human nuclei antibody staining demonstrated that all three cells types survived for the 2-week period. The DSC were found in close proximity to the SC at the proximal regeneration front (Figure 5B). The S-100 staining suggested that the DSC themselves did not differentiate into SC (See paper II, Fig. 5B).

9.2 Effects of stem cells on the reactions in DRGs and spinal cord

qRT-PCR analysis of the sensory L4-L6 DRG neurones and corresponding motor neurone 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).

Caspase-3 levels in the DRG were used as a measure of the viability of the sensory neurones. Compared with control DRG, the DRG harvested from sciatic nerve injured rats treated with empty conduits showed a 3-fold increase in caspase-3 expression levels (See paper II, Fig 5D). Conduits seeded with any of the four cell types significantly reduced caspase-3 expression compared with the empty conduit group (See paper II, Figure 5D), suggesting a neuroprotective action, in addition to the effects on axon regeneration.

9.3 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/mm^2 in conduits without cells to 26.49±3.66/mm^2 ($P < 0.05$) in ASC seeded conduits and 34.55±4.86/mm2 ($P < 0.01$) in stimulated ASC-loaded conduits (See Paper I, Fig. 7B).

10. TRANSPLANTATION OF HUMAN ASC INTO INJURED SPINAL CORD

10.1 Survival of transplanted ASC

One week after SCI and ASC transplantation with CsA treatment, numerous cells immunolabelled for human nuclear antigen (HNA; See Paper III, 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 III, Fig. 4B) and at 3 weeks, only single HNA-labelled cells were found in the trauma zone and injection sites (See Paper III, 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 III, 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 (Paper III, Fig. 4A-C).

10.2 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 *angiopoietin-1* one week after transplantation (See Paper III, 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 III, 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 III, Fig. 5A,B). The labelling was significantly decreased 2 weeks after ASC transplantation (See Paper III, Fig 5D,E). After transplantation, ASC continued to express laminin both at 1 week and 2 weeks postoperatively (See Paper III, 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 III, Fig. 5G,H) but at 2 weeks postoperatively it was increased throughout C3-C4 spinal cord segments (See Paper III, Fig. 5I,J). However, the pattern of neurocan expression around ASC injection sites was changed when compared with 1 week transplantation site (See Paper III, 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 III, 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 III, 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 III, Fig. 6).

10.3 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 III, 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 III, 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 III, Fig. 7F) but had no effect on the contralateral side (See Paper III, 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 III, 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 III, 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 III, 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 III, Fig. 7H) and two-fold increase on the contralateral side (P<0.01; See Paper III, 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 III, Fig. 7H,I).

10.4 Effects of ASC 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 III, Fig. 8A). Transplantation of ASC cranial and caudal to the site of injury changed the structure of the astroglial scar (See Paper III, 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 III, 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 III, Fig. 9A and Fig. 8D, E) and OX42-positive microglia (P<0.001; See Paper III, Fig. 9B and Fig. 8G, H). In addition, SCI increased the density of RECA-1 positive vascular endothelium by 60% (See Paper III, Fig. 9C). Transplantation of ASC significantly attenuated the astroglial and microglial cell reactivity (P<0.001; See Paper III, Fig. 9A, B) but had no effect on the vascular endothelium (P<0.05; See Paper III, Fig. 9C).

10.5 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 III, Fig. 10).

11. TRANSPLANTATION OF POLYPEX PARTICLES INTO THE SPINAL CORD

11.1 Polyplex particles are internalised by microglia and macrophages and localise to the SCI zone

Three to seventy-two hours post microinjections of chitosan formulated siRNA-Cy3 particles into C3-C4 segments of normal spinal cord, the Cy3 signal could be detected at the injection sites (See paper IV, Figure 5A, D, G) in the form of small bright round spots (See paper IV, insertion in Figure 5A) and as a diffuse staining of the surrounding spinal cord parenchyma. The siRNA-Cy3 signal, appears to increase within the first 48 h (data not shown), this may be due to scavenging activity of activated macrophages and microglial cells concentrating the particles in endosomes.

Additional immunostaining with antibodies against glial and neuronal markers (See paper IV, Figure 5B, E, H) revealed that OX42 positive macrophages and microglial cells have a preference to internalize the particles (See paper IV, Figure 5B, C). In contrast, the GFAP positive processes of astrocytes (See paper IV, Figure 5E, F) and NeuN positive neuronal cell bodies (See paper IV, Figure 5H, I) did not show any co-localisation with Cy3. Three days after C3 spinal cord hemisection and intraperitoneal injection of chitosan formulated siRNA-Cy3 particles, small clusters of Cy3/ED-1 positive activated macrophages were found within 300-500μm rostral and caudal to the injury site (See paper IV, Figure 5J-L). The Cy3 signal in these blood-born macrophages appeared both as granular and diffuse staining (See paper IV, Figure 5J).

11.2 Effects of miR-124 chitosan particles on macrophage/microglia reaction

Microinjection of 1μL chitosan formulated miR-CTRL particles into intact cervical C3 spinal cord of adult rats induced activation of ED-1 positive macrophages and microglial cells along the injury canal left after withdrawal of the 70μm thick tip of the glass micropipette (See paper IV, Figure 6 A, C). In contrast, injection of particles with miR-124 reduced the number of ED-1 labelled cells by 80% ($P<0.001$; See paper IV, Figure 6 B, C). In the experiments with spinal cord injury, the rats underwent cervical C3 hemisection and chitosan formulated miR-CTRL particles were injected 1 mm

rostral and caudal to the injury site. The reaction of ED-1 positive macrophages and microglial cells was measured in the trauma zone. Injections of miR-124 containing particles reduced the number of ED-1 positive cells by 60% ($P<0.001$; See paper IV, Figure 7). However, when miR-124 particles were introduced into the spinal cord cavity within a small piece of Spongostan®, no reduction in macrophage and microglia activity was found (data not shown).

DISCUSSION

12. PRODUCTION OF GROWTH FACTORS BY HUMAN STEM CELLS

A multifactorial approach is needed to deal with the numerous challenges following SCI and PNI. Stem cells can be one important element of this since they can respond concurrent to the fluctuating demands after injury. A number of different mechanisms have been proposed regarding the role stem cells have after transplantation [5,135,136]. 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 neurones; remyelination of spared axons; bridging of cysts or cavities (acting as a bioactive scaffold) and stimulation of endogenous precursor cells. In this thesis, the aim was to determine through which mechanisms both human ASC and DSC would exert their effects in SCI and PNI experimental models, and the effects of targeted suppression of the neuroinflammatory response using miRNA-124 following SCI.

Papers I, II and III showed that the stem cells produced 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. In contrast, stimulation of the DSC populations (SCAP, DPSC & PDLSC) only resulted in significant increases in BDNF and VEGF-A expression. Subsequent *in vitro* effects on neurogenesis or angiogenesis were also limited.

It has previously been demonstrated that BDNF, when applied via an osmotic pump [137] or when produced by transplanted bone marrow stem cells [101], provides beneficial effects after spinal cord injury. BDNF has been described to induce plasticity in the injured spinal cord and plays a role in axonal myelination [138-140]. FGF-2 has

been shown to protect CNS neurones, reducing the effects of anoxia, hypoglycaemia, excitatory amino acids, free radicals, excess intracellular calcium, and nitric oxide [141-144], thereby decreasing the need for macrophage/microglia recruitment. Exogenously applied FGF-2 has been shown to lead to physical alignment of astrocytes [145]. 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 [146,147].

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 neurones 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 [148]. The same research group compared the *in vivo* trophic factor expression levels produced by transplanting NPCs or bone marrow MSC [149]. 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 [149]. 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 [150-153]. Comparisons have shown that ASC produce significantly higher amounts of growth factors like VEGF, HGF and BDNF when compared to bone marrow MSC [154-156].

In Paper I it was demonstrated that growth factor stimulation of the ASC resulted in increased neurotrophic factor gene expression and consequent enhanced levels of 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 [157] whilst FGF-2 enhances BDNF expression in retinal ganglion cells [158]. Previously it was shown that the same mixture

of factors could induce the expression of glial cell proteins in rat ASC [124]. The elevated secretion of neurotrophic factors was consistent with increased neurite outgrowth of DRG neurones exposed to conditioned medium from the stem cells. Human bone marrow MSC also enhance DRG neurite outgrowth but most likely independently of BDNF [159]. Other studies have shown that human ASC express a range of neurotrophic factors, which can enhance neurite outgrowth of neuronal cell lines [160-162].

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 [163]. When ASC are cultured under hypoxic conditions they secrete increased levels of VEGF and can convert to an endothelial cell phenotype [164,165]. ASC are often genetically modified to over-express VEGF [166,167] 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* [166]. Unstimulated ASC could promote HUVEC capillary-like formation and 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 [168]. The growth factors FGF-2 and PDGF used in the stimulation protocol are the most likely to control the production of VEGF since there is significant cross-talk between all three molecules [169]. In addition to the secretion of VEGF elevated levels of angiopoietin-1 were detected in the stimulated ASC medium. It has been previously shown that rat ASC express angiopoietin-1 [170] 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 angiopoietin [171]. In contrast, another study showed that it was necessary to genetically modify the ASC to enable significant secretion of angiopoietin [172]. In the context of Paper I it is also interesting to note that adipocyte derived angiopoietin-1 supports neurite outgrowth and synaptogenesis of sensory neurones [173].

13. ASC AND DSC PROMOTE AXONAL REGENERATION AFTER PNI

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 the first report of Schwann cell-like differentiation of rat ASC [124] a number of research groups have investigated the effect of rat ASC on peripheral nerve regeneration. Schwann cell-like differentiated ASC enhance motor neurone regeneration and improve evoked electrical potentials in the gastrocnemius muscle [108] and also at early stages of regeneration they down-regulate apoptotic markers in the DRG suggestive of a pro-survival effect [174]. Undifferentiated rat ASC have also been shown to enhance peripheral nerve repair [175,176] and they may act synergistically with Schwann cells to secrete growth factors such as NGF [177]. Alternatively the stem cells can be neurally induced to release enhanced levels of BDNF, which is necessary for their nerve stimulatory effects [178]. 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 [179]. 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 [180]. Furthermore in that study there was no evidence of stem cell trans-differentiation *in vivo* [180] which is in contrast to the recent report by Tomita et al. [162] 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 [181].

Recent studies in mice have shown that DSC can develop from glial cells [182]. A number of different DSC populations have been cultured from the teeth including stem cells from human exfoliated deciduous teeth (SHED) and periodontal ligament of deciduous teeth stem cells (DePDL) which are present in deciduous teeth; SCAP and dental follicle progenitor cells (DFPC) found in developing teeth and DPSC and PDLSC found in permanent teeth [183]. In paper III, SCAP, DPSC and PDLSC populations were isolated from 2 individuals and experiments showed that they exhibited properties of mesenchymal stem cells [184-186].

Of these cell types, DPSC have been the most commonly used in models of nerve injury [184,187,188], with a few studies investigating other DSC [189,190]. The results in paper II also show that human DPSC and PDLSC significantly improve the regenerative process following peripheral nerve injury. In addition, for the first time, it was demonstrated that SCAP significantly improve the distance of axonal regeneration compared to both DPSC and PDLSC. Although not compared directly, the results suggest that human SCAP increase nerve regeneration to a greater extent than human adipose stem cells [134]. These findings are consistent with DPSC-enhanced *in vitro* neuroprotection and neurogenesis on retinal ganglion cells when compared with bone marrow and adipose MSCs [191].

14. 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. Therefore the effects of stem cell transplantation on the expression of a number of genes known to be affected by peripheral nerve injury were examined. Growth associated protein *GAP-43* is a molecule up-regulated in sciatic motor neurones and L4-L6 DRG neurones following sciatic nerve injury [192] and has been implicated as one of several important mediators of peripheral nerve regeneration [193]. Typically treatments such as electrical stimulation and growth factor administration which stimulate regeneration are associated with elevated levels of *GAP-43* [62,194]. 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 neurone regeneration to a greater extent than sensory neurones. A number of studies have shown that peripheral nerve axotomy leads to DRG sensory neurone cell death [195,196]. In Paper I it was shown that stimulated human ASC 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. Similar effects were found for the DSC. This is consistent with studies using rat ASC [174] and mouse models [197]. Regeneration and apoptosis associated genes are controlled by multiple signalling pathways and in Paper I the expression levels of *ATF-3* and *c-jun* were determined. ATF-3 is rapidly induced in DRG sensory neurones and motor neurones following axotomy [198]. ATF-3 increases the intrinsic growth state of DRG neurones to enhance peripheral nerve regeneration [199] and in the central nervous system, regenerating neurones are associated with increased ATF-3 expression [200]. 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 [199] but GAP-43 expression levels are higher in ATF-3 positive neurones after electrical stimulation [194]. In the DRG it has been shown that non-regenerating neurones fail to up-regulate ATF-3 [201]. It is therefore possible that ASC boosted the number of regenerating motor neurones 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 [202]. 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 [203]. 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 [204]. Further studies in which neurone specific c-jun was deleted showed similar effects [205]. Consistent with previous reports [206], the results in Paper I showed that *c-jun* was up-regulated in the injured DRG and when stem cells

were transplanted 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 the stimulation protocol has been shown to reduce injury-induced increases in c-jun immunoreactivity [207]. Conversely, inhibition of c-jun phosphorylation has been shown to reduce axonal outgrowth of DRG neurones [208]. Similar anomalies have been reported when studying c-jun expression in the motor neurones. Increased levels of phosphorylated c-jun are observed in distally axotomised motor neurones of neonatal rats but this is not the case for adult rats [209]. However, avulsion injury in adult rats does result in increased phospho c-jun which is associated with significant cell death [209]. A long term association of phosphorylated c-jun with ATF-3 in axotomised motor neurones correlates with degenerating neurones [210]. In other studies, decreased c-jun expression has been correlated with impaired motor neurone regeneration in aged animals [211]. In Paper I it was found that nerve injury alone down-regulated *c-jun* levels in the spinal cord and this might protect motor neurones against axotomy induced cell death. Nevertheless, how decreased *c-jun* levels can be correlated with the other observations of increased *GAP-43* expression and improved regeneration in the periphery remains to be elucidated. It should be noted that these molecules have been examined just at one time point in Paper I and the interaction between the various transcription factors and regeneration-associated genes is likely to be a very dynamic process following injury.

15. EFFECTS OF ASC ON EXTRACELLULAR MATRIX MOLECULES

There are no previous reports showing stem cells acting by reducing chondroitin sulphate proteoglycan (CSPG) production. CSPGs form a perineuronal 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 [212] and found a decrease in neurocan immunostaining around the cell transplantation sites. Results in paper III showed significant sprouting and ingrowth into the 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 [213]. It is also possible that 5HT sprouting was supported by continuous expression of laminin [214], which has been shown to aid serotonergic sprouting following spinal injury.

16. IMMUNOSUPPRESSIVE TREATMENT & HUMAN STEM CELL TRANSPLANTATION

A number of studies have shown the survival of human MSC *in vivo* with [156,215] or without [216,217] immunosuppression. We found CsA was required for human cell survival, as has been previously reported when using human bone marrow MSC in the rat sciatic nerve injury model [130]. The loss of ASC is accelerated by recruitment of the adaptive immune response to aid innate immunity [218]. It has been shown that these effects are reduced when CsA is administered [219] 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. [220], 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 [221] and B cell function [222] and thereby modulate the inflammatory reaction after injury. Interestingly human ASC can also facilitate the immunosuppressive effects of CsA [220] so could potentially be used to reduce the adverse effects of CsA which is a requirement for effective nerve allografting.

17. ASC STIMULATE AXONAL SPROUTING AFTER SCI

Current literature suggests that CNS axons sprout in the region of the scar [223], in an attempt to bridge it [224], 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 [101,131,140,149]. In our spinal cord injury model, ASC transplantation significantly decreased sprouting of the descending serotonergic fibres in C3 segment on the injured side. This may occur due to the cumulative effect of a number of factors. Firstly, improved survival of the cranially placed neurones 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 [145,225,226], as a result of decreased microglial activity, decreased neurocan production [26] and increased FGF-2 release [227]. Remodelling of the nervous system occurred with contralateral sprouting of the serotonergic axons at C2 spinal cord level possibly through the decreased CSPG production [213] and retrograde transport of growth-promoting molecules. 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 [228]. This pattern of plasticity in the serotonergic system has been shown to correlate with improved functional outcomes [229]. This is the first time that this has been described with ASC, although similar effects have been shown after bone marrow MSC transplantation [230].

18. EFFECTS OF ASC ON GLIAL CELL REACTIONS

Astrocytes play a key role in the pathophysiology after SCI [231], leading to decreased production of growth factors and increased expression of inhibitory molecules [232]. Reduced astrocyte activation may occur as a result of early inactivation of microglia [225,233], although the interaction is complex and reduced levels of CSPGs have been shown to decrease microglial cell activity [231]. The results in Paper III 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 [101,131,140].

Secondary damage in SCI is propagated by CNS glial cells and cells infiltrating from the peripheral circulation [23]. Together, they seal off the injured area, decrease pro-

growth factors, and increase extrinsic inhibitory molecule production [223]. A recent report also demonstrates that blood derived macrophages can facilitate secondary axonal dieback after spinal cord injury [234]. Although the data showing 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.

19. 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 [235] and nerve conduits that facilitate early vascularisation are superior to simple silicone conduits [236]. Enhanced angiogenesis has been suggested to be one beneficial effect of MSC transplantation [146,156,237,238]. VEGF-A and angiopoietin-1 released from ASC in SCI could decrease damage to the neurones and axons, preventing demyelination, which may reduce the need of the system to induce neovascularisation [146]. The results in Paper I showed 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 [239,240]. The studies of ASC transplanted into the injured spinal cord in Paper III also demonstrated that ASC can produce angiopoietin-1 and VEGF-A for at least 3 weeks postoperatively. However, in contrast to peripheral nerve injury, the results did not show any effect on neovascularisation after SCI at 8 week postoperatively.

20. miRNA-124 REDUCES THE INFLAMMATORY LOAD AFTER SCI

Experiments in Paper IV demonstrated that miR-124 transfection reduces MHC-II, TNF-α and ROS production in bone marrow derived macrophages, in support of a

previous study in mice [115]. While microinjections present a convenient model of CNS inflammation simply due to the physical disruption of the BBB by the needle cavity, the clinical application of such an invasive technique is minimal. Disruption of the BBB during traumatic injury, Alzheimer's disease [241], MS [242], and Parkinson's disease [243] leads to a host of complications for CNS repair, but can also provide an avenue for infiltration of therapeutics from the blood stream. By loading macrophages with particles containing catalase, and injecting them intraperitoneally into an induced mouse model of Parkinson's disease, particle loaded macrophages have been shown to localize in the affected midbrain and protect neurones by reducing ROS [244].

The miR-124 particle treatment was effective in reducing local ED-1 positive cells in an acute *in vivo* SCI model. Long-term experiments are needed to study the effect miR-124/ chitosan particles may have on the secondary tissue disruption associated with SCI. It is certainly conceivable from the evidence presented that the marked reduction of ED-1 expressing macrophages/microglia in miR-124 treated SCIs correlate with a reduction in inflammatory secretome expression.

Polarization of microglia/macrophages to the activated M1 phenotype is linked to the sustained inflammatory conditions of neurodegenerative diseases; the alternatively activated M2 phenotype performs the opposite function and enhances neuroprotection and regeneration in SCI [245]. Therefore the strategy employed, to reduce neurotoxic agents in the CNS by delivering miR-124 to induce macrophage quiescence, has the potential to be enhanced further by exploring the possibility of promoting M2 polarization over that of M1. As shown in paper IV, delivery of miRNA/particles to M1 activated macrophages/microglia and the subsequent reduction of negative effects thereof, a similar strategy could be employed to deliver a miRNA responsible for promoting the transition of M1 to M2 polarization or by delivering an anti-miR to reduce the effect of an M1 specific miRNAs. An anti-miR towards miR-155 could also be a viable option for delivery to SCIs as the miRNA has been shown to promote M1 polarization in human macrophages while its inhibition blunts the expression of pro-inflammatory cytokines in a MS model [246]. It has recently been shown that miRNA let-7c promotes M2 polarization by regulating C/EBP-δ in mouse bone marrow

derived macrophages and is conversely associated with M1 polarization upon silencing using anti-miRs [247], and may thus be an alternate path of investigation.

21. CHITOSAN PARTICLES ARE A SUITABLE METHOD OF DELIVERING miRNA

Chitosan particles have previously been found to be transported with recruited macrophages to sites of inflammation in a mouse models of rheumatoid arthritis [248], radiation induced fibrosis [249,250], kidney fibrosis [251], and periodontal lesions [252]. To evaluate if the same mode of delivery is available for use in future CNS inflammation studies, in paper IV we performed SCIs, followed by intraperitoneal injection of chitosan particles to assess the recruitment of the particle loaded macrophages to the injury site. The migration of peritoneal macrophages to the spinal cord lesion was validated by the detection of internalized chitosan/siRNA-Cy3 particles. It remains to be seen if miR-124 can have the same effect on macrophage activation in SCIs if delivered to the peritoneum, as these cells may not be as motile when quiescent [253,254]. Additionally, motility may directly be affected by miR-124 targeting STAT3, as this signalling pathway has been shown to regulate cell movement in keratinocytes, leukocytes, and epidermal cells [255].

Regardless of the cargo, this delivery strategy presents a promising, minimally invasive means to address CNS inflammation in future studies and may also provide a method of quantifying and studying the effects and the resident vs. migratory macrophage population in such conditions.

CONCLUSIONS

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

- Human mesenchymal stem cells derived from adipose (ASC) and dental (DSC) tissue express various neurotrophic and angiogenic molecules. Growth factor stimulation of the stem cells resulted in increased secretion of these proteins and promoted neurite outgrowth from adult rat sensory DRG neurones. Stimulated ASC also showed an enhanced ability to induce capillary-like tube formation in an *in vitro* angiogenesis assay.

- In a peripheral nerve injury model, ASC and DSC promote axonal regeneration in the conduit and reduced caspase-3 expression in the DRG. ASC also enhanced GAP-43 and ATF-3 expression in the spinal cord, reduced c-jun expression in the DRG and increased the vascularity of the implant.

- ASC do not induce activation of astrocytes and after transplantation into injured spinal cord continue to express growth factors when combined with immunosuppression treatment. Transplanted cells induce sprouting of serotonergic raphaespinal axons, reorganise the astrocytic network around the lesion site and attenuate the astrocyte and microglial cell reactivity.

- Upon microinjection into uninjured rat spinal cords, particles formed with Cy3-labeled control sequence RNA, were specifically internalized by OX42 positive macrophages and microglia. Microinjections of chitosan/microRNA-124 particles into injured spinal cord significantly reduced the number of ED-1 positive macrophages.

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