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Mesenchymal stem cells for repair of the peripheral and central nervous system

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Cover illustration: Differentiated human mesenchymal stem cells in culture/
pastel crayon layout

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSC) have been shown to provide neuroprotection after transplantation into the injured nervous system. The present thesis investigates whether adult human and rat MSC differentiated along a Schwann cell lineage could increase their expression of neurotrophic factors and promote regeneration after transplantation into the injured peripheral nerve and spinal cord.

Human and rat mesenchymal stem cells (hMSC and rMSC) expressed characteristic stem cell surface markers, mRNA transcripts for different neurotrophic factors and demonstrated multi-lineage differentiation potential. Following treatment with a cocktail of growth factors, the hMSC and rMSC expressed typical Schwann cells markers at both the transcriptional and translational level and significantly increased production of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF).

Age and time in culture are of relevance for clinical settings and growth-promoting effects of hMSC from young donors (16-18 years) and old donors (67-75 years) were compared. Undifferentiated hMSC from both young and old donors increased total neurite length of cultured dorsal root ganglion (DRG) neurons. Differentiation of hMSC from the young donors, but not the elderly donors, further enhanced the neurite outgrowth. Undifferentiated hMSC were cultured for eleven weeks in order to examine the effect of in vitro expansion time on neurite outgrowth. hMSC from the young donors maintained their proliferation rate and their ability to enhance neurite outgrowth from DRG neurons.

Using a sciatic nerve injury model, a 10mm gap was bridged with either an empty tubular fibrin glue conduit, or conduits containing hMSC, with and without cyclosporine treatment. Cells were labeled with PKH26 prior to transplantation. At 3 weeks after injury the conduits with cells and immunosuppression increased regeneration compared with an empty conduit. PKH26 labeled human cells survived in the rat model and the inflammatory reaction could be suppressed by cyclosporine.

After cervical C4 hemisection, BrdU/GFP-labeled rMSC were injected into the lateral funiculus rostral and caudal to the spinal cord lesion site. Spinal cords were analyzed 2-8 weeks after transplantation. Transplanted MSC remained at the injection sites and in the trauma zone for several weeks and were often associated with numerous neurofilament-positive axons. Transplanted rMSC induced up-regulation of vascular endothelial growth factor in spinal cord tissue rostral to the injury site, but did not affect expression of brain-derived neurotrophic factor. Although rMSC provided neuroprotection for rubrospinal neurons and significantly attenuated astroglial and microglial reaction, cell transplantation caused aberrant sprouting of calcitonin gene-related peptide immunostained sensory axons in the dorsal horn.

In summary these results demonstrate that both rat and human MSC can be differentiated towards the glial cell lineage, and show functional characteristics similar to Schwann cells. hMSC from the young donors represent a more favorable source for neurotransplantation since they maintain proliferation rate and preserve their growth-promoting effects in long-term cultures. The data also suggest that differentiated MSC increase expression of neurotrophic factors and support regeneration after peripheral nerve and spinal cord injury.

Key words: Bone marrow-derived stromal cells, Schwann cells, Peripheral nerve injury, Spinal cord injury, Neurotransplantation

ORIGINAL PAPERS

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

- I. Maria Brohlin, Daljeet Mahay , Lev N. Novikov , Giorgio Terenghi , Mikael Wiberg , Susan G. Shawcross and Liudmila N. Novikova. Characterisation of human mesenchymal stem cells following differentiation into Schwann cell-like cells. *Neuroscience Research*, 64,41-49, 2009.
- II. Maria Brohlin, Paul J. Kingham, Liudmila N. Novikova, Lev N. Novikov, Mikael Wiberg. Aging effect on neurotrophic activity of human mesenchymal stem cells. Submitted *Neuroscience Research*.
- III. Aleksandra M. McGrath*, Maria Brohlin*, Paul J Kingham, Lev N. Novikov, Mikael Wiberg and Liudmila N. Novikova. Fibrin conduit supplemented with human mesenchymal stem cells supports regeneration after peripheral nerve injury. Manuscript.
- IV. Liudmila N. Novikova, Maria Brohlin, Paul J Kingham, Lev N. Novikov and Mikael Wiberg. Neuroprotective and growth-promoting effects of bone marrow stromal cells after cervical spinal cord injury in adult rats. *Cytotherapy*, 13,873-887, 2011.

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SVENSK SAMMANFATTNING

Att få en perifer nervskada, dvs. en skada i armen eller i handen, eller en central skada i ryggraden är en stor ekonomisk börda för samhället, både direkt men även genom bortfall av arbetskraft. Känselbortfall och nedsatt rörelse medför en sämre livskvalitet för patienten. Vid operationer idag används en nerv från benet för att sy ihop skadan men det leder i sin tur till försämrad nervfunktion även i detta område. Kroppens egna gliaceller, Schwannceller (SC), som naturligt ligger runt nerven hjälper till att läka skadan genom att bland annat skicka ut olika tillväxtfaktorer. Schwannceller är svåra att odla fram och i denna studie används mogna mesenkymala stamceller (MSC) som alternativ. Dessa celler har visat sig ge en nervskyddande effekt på det skadade nervsystemet efter transplantation. Denna forskning med cellterapi kan leda till en större förståelse och bidra till nya möjligheter för läkning av nervskador i det perifera och centrala nervsystemet.

Mesenkymala stamceller behandlas med tillväxtfaktorer och analyseras på gen-och protein nivå, men framförallt studeras dess funktionella egenskaper, dvs. dess förmåga att generera nervtillväxt i skadeområdet. Celler transplanteras i en skadad perifer nerv samt i en skadad ryggrad och vi kunde påvisa cellöverlevnad och nervtillväxt.

Denna studie ger positiva indikationer för att stamceller kan användas som alternativ vid behandlingen av läkning på nervskador i det perifera och centrala nervsystemet.

ABBREVIATIONS

α MEM	α -Minimum Essential Medium
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DMEM	Dulbecco's modified eagle medium
dMSC	Differentiated mesenchymal stem cell
DRG	Dorsal root ganglia
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GDNF	Glial-derived neurotropic factor
GFAP	Glial fibrillary acidic protein
GGF-2	Glial growth factor-2
MSC	Mesenchymal stem cells
NGF	Nerve growth factor
NT-3	Neurotrophin-3
P	Patient
PDGF-AA	Platelet-derived growth factor-AA
PE	Phycoerythrin
PNS	Peripheral nervous system
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Schwann cells
SCI	Spinal cord injury
VEGF	Vascular endothelial growth factor

INTRODUCTION

Clinical background

Peripheral nerve injuries (PNI) and spinal cord injuries (SCI) are injuries that cause loss of sensory and motor function. Several hundred thousand patients are affected every year in Europe. The injuries are most common in young adults and often caused by traffic accidents, occupational trauma with machinery knives and glass, assaults and self-destructive behavior (Asplund et al. 2009), (Rosberg et al. 2005), (van den Berg et al. 2010). This is an economic burden for the society and the patients attain poor sensory and reduced motor functions (Wiberg and Terenghi 2003). The cost per patient for PNI is estimated to be 51338 euro for ulnar nerve and 31168 euro for median nerve (Rosberg et al. 2005). In the United States the annual cost for SCI is estimated to be seven billion dollars (McDonald and Sadowsky 2002). Approximately 2.5 million people worldwide are suffering in total from traumatic SCI (Thuret et al. 2006). In Sweden, approximately 120 individuals, mostly young male adults, are injured on an annual basis (Holtz A and Levi R 2006). At the moment there are no effective treatments for SCI but for PNI the nerve ends can be sutured together with advanced microsurgical techniques with some functional recovery (Wiberg and Terenghi 2003).

Peripheral nerve injury

The most common injuries are transection, laceration and compression and to classify them in a clinical settings they are placed in three categories named neuropraxia, axonotmesis and neurotmesis (Seddon et al. 1943). Neuropraxia is the mildest form where the nerve is anatomically intact but the propagation of impulses are disrupted. The most severe injury is the neurotmesis where the axon, myelin sheaths and the connective tissue are disrupted and without surgical treatment there is no functional recovery (Seddon et al. 1943). An injury where the myelin sheaths and several nerve fibers are damaged with minimal connective tissue trauma is called axontomesis. The peripheral nerve injuries affect both the sensory and motor neurons. Only 50% of sensory neurons in the dorsal root ganglion (DRG) survive after a transection of the peripheral nerves in adult rodents (Welin et al. 2008). Primary repair could rescue some of the injured sensory neurons. Motor neuron cell death is minimal but still only 55-59% axons regenerate after primary nerve repair (Welin et al. 2008). Schwann cells are the essential glial cells which surround the axons, produce myelin sheaths that wraps around the axon and provide neurotrophic growth factors following an injury (Bunge 1994), (Jessen and Mirsky 1999). The process called Wallerian degeneration, a complex cascade of events, is initiated in the distal nerve and is completed within two months after an injury occurs. The cascade involves activation of macrophages to remove debris, myelin degradation and degeneration of axons. This process is of importance for the regeneration of the surviving neurons (Navarro et al. 2007). The Schwann cells proliferate and line up to create the bands of Bungner which provide the guidance for the regenerating axons. They increase BDNF, LIF, NGF and NT4 secretion and up-regulate GGF receptor mRNA as well as cytokines and laminin (Terenghi 1999), (Hall 2001).

Spinal cord injury

Massive compression caused by laceration, solid cord injury and contusion are groups that classify the spinal cord trauma defined by the “Miami Project to Cure Paralysis”. Half of the injuries are caused by massive compression with laceration, one third compression and the final 17% represent the solid cord injury. Solid cord injury keeps the cord intact and the changes may be detected with microscopical techniques. Laceration and compression damages the spinal cord parenchyma and the meninges (Harper et al. 1996), (Bunge and Pearse 2003), (Tator 2006). The tissue response after a spinal cord injury is divided into four phases: the immediate phase, the acute phase, the intermediate phase (phase of secondary tissue loss) and the chronic (late) phase.

The initial immediate phase lasts for several hours, causes hemorrhage followed by an inflammatory response mainly in the gray matter resulting in hypoxia and lack of glucose to the neurons. This initial phase affects the neurons, astrocytes and oligodendrocytes by triggering necrotic and apoptic cell death and demyelination and degeneration of the axons (Quencer 2002), (Donnelly and Popovich 2008) .

The primary lesion also triggers a cascade of secondary degenerative changes that lead to cavitation and glial scar formation in the lesion zone due to the release of toxic amino acids and metabolic stress. Astrocytes proliferate in response to inflammatory cytokines and form the glial scar. Proteoglycans expression in the scar inhibits the axon regeneration. This secondary intermediate phase lasts from a few minutes up to days (Bareyre and Schwab 2003) and the late phase lasts weeks or years after the trauma. In the last and chronic phase Wallerian degradation occurs. Myelin is speculated to also act as a major source of inhibitory molecules that prohibits axon regeneration and blocking myelin proteins results in better regeneration (Schwab 2004), (Xie and Zheng 2008). Initial trauma and secondary chronic phase both results in sensory and motor function loss due to lesions in descending and ascending pathways together with the intrinsic circuitry of the spinal cord (Tator 1995;Tator and Fehlings 1991).

Loss of nerve tissue and bridging strategies

Since both spinal cord and peripheral nerve injury in humans may result in a significant loss of nervous tissue, bridging the defects with growth-permissive materials is essential for future potential therapies. Peripheral nerve injuries may cause loss of nerve tissue but trauma with short gaps are often treated by direct epineural suturing. If the gap is longer, a bridging nerve graft of some type is needed. The golden standard is the autologous nerve graft, using a sensory nerve usually from the patient’s own leg, but this causes scarring, loss of sensation and sometimes pain in the donor region (Wiberg and Terenghi 2003). To overcome these problems new biosynthetic conduits has been investigated in order to replace the nerve autografts and allografts (Evans et al. 1994). The problem is to find an inert biodegradable material that will support the regenerating axons and suppress immunological rejections (Novikova et al. 2003). Attempts have been made using non-degradable materials as silicone (Merle et al. 1989), (Lundborg et al. 1991), PTFE (polytetraflourethylene) and PPY (polypyrrole) (Gibson et al. 1991) but the supportive effects do not gain the axon regeneration nor survival often due to compression (Mohanna et al. 2005). We have in our group shown that PHB (poly-3-

hydroxybutyrate), a biodegradable material, could be used for transplantation both for clinical work (Aberg et al. 2009) as well as for experimental studies (Novikov et al. 2002). The PHB is easy handable and has a unidirectional fiber orientation and is resorbed within 24 months (Young et al. 1980). Although PHB is clinically applicable the search for other materials that have the possibility to degrade faster and make a better environment for neurotrophic factor support, myelination and guidance by endogenous glia cells (reviewed in (Jiang et al. 2010) ; (Novikova et al. 2003) is continuing. Fibrin glue is well known by surgeons in reconstructive surgery and has shown good scaffold abilities in tissue engineering and nerve regeneration (Kalbermatten et al. 2008). To enhance the growth-promoting support for the regenerating axons addition of various types of cells are required to give the best milieu by secreting neurotrophic factors and promoting revascularization.

Cells for transplantation

Schwann cells produce neurotrophic factors, extracellular matrix molecules and associated integrins that promote the growth of the nerve (Terenghi 1999), (Bunge and Pearse 2003). During peripheral nerve injury NGF and BDNF are produced by Schwann cells which leads to activation of intracellular signal transduction through tyrosine kinase (trk) receptors. This effect stimulates neuronal survival and axonal regeneration. The neurotrophic factors belong to the groups of peptides that affects the survival and development of neurons and axon outgrowth (Terenghi 1999). These factors are produced by the target organs and transported by retrograde transport. NGF binds to trkA receptors on the neurons while BDNF binds to trkB receptors (Lindsay 1996). Following axotomy, BDNF expression is initiated after 4 days and achieves peak levels after 4 weeks but NGF, in the distal Schwann cells, has its highest levels after 24h and remains elevated for 14 days (Ide 1996). BDNF has been shown to reduce motor neuron death after ventral root avulsion in adult rats (Novikov et al. 1995), (Novikova et al 1997). VEGF is also of importance since it promotes axon regeneration either directly or by stimulating angiogenesis (Hobson et al. 2000), (Pons et al. 2008). Integrins are membrane proteins that anchor cell matrix interactions and are up-regulated by BDNF and NT3. This enhances the regeneration of neurons (Plantman et al. 2005). Extracellular matrix molecules, fibronectin and laminin are synthesized on the basement membrane to increase the surface cell adhesion molecules (CAMs).

Schwann cells can be cultured *in vitro*, and their transplantation within a nerve conduit has shown promising results for axonal regeneration in both peripheral and central nervous system injuries (di Summa et al. 2011), (Shimizu et al. 2007), (Kamada et al. 2010), (Kocsis et al. 2004). However, the use of autologous cultured Schwann cells for the treatment of acute injuries may be impractical due to the technical difficulties and the time required in harvesting and expanding the slow growing Schwann cells in order to obtain a sufficient number of Schwann cells for transplantation into a conduit (Mosahebi et al. 2001). Such a delay in carrying out the nerve repair following injury would be deleterious to the clinical outcome, since increased time between injury and repair results in a more neuronal cell death, and thus less chance for functional recovery (Hart et al. 2002).

Since Schwann cells have limited clinical applicability, much attention has been given to the identification of other potential sources of transplantable cells. These cells should ideally be easily accessible, have rapid expansion profiles in culture, be immunological inert, and capable of long-term survival and integration in the host tissue. The demonstration that both embryonic stem cells as well as stem cells isolated from adult tissues are able to differentiate into neural/glial cell types and to promote neural regeneration has led to a rapidly evolving field for stem cell based therapies for the treatment of degenerative or traumatic lesions of the nervous system. Stem cells are cells that can divide and give rise to different kind of cells in the organism. Embryonic stem cells are derived from the inner cell mass of the blastocyst and can differentiate into all cell types. Following injection into the blastocyte they can divide and form three germ layers and differentiate both spontaneously and under the influence from different chemicals into specific cells. They are totipotent during early cell divisions but pluripotent in a later state. However, there are both medical and ethical dilemmas associated with embryonic stem cells. They are immature and divide rapidly and might give teratomas. The tissue origin, legislation, and religion in different countries are other factors of importance for the patient (Hei 2010), (McCormick and Huso 2010).

Mesenchymal stem cells (MSC) are derived from many different sources such as bone marrow, adipose tissue, skin and liver (Krampera et al. 2007) reviewed in (Barry and Murphy 2004)). They are adult stromal cells that are multipotent and they have a more restricted differentiation than embryonic stem cells. There are no specific markers for identification of the MSC and that makes the absolute identification and characterization of the cells difficult. However, the International Society of Cellular Therapy (ISCT) states that human MSC must be positive for certain markers as well as show differentiation properties to different lineages (Dominici et al. 2006), (Horwitz 2003). MSC are spindle shaped, flattened, fibroblast like cells that were first identified by Friedenstein in the 1970's (Friedenstein et al. 1974). The population is very heterogenous containing less than 0.1% of stem cells that may differentiate into chondrocytes, oestoblasts, adipocytes. They are however easily harvested and cultured as adherent cells under special conditions *in vitro* for gaining sufficient number of cells for transplantation. Until recently, dogma dictated that organ-specific stem cells were restricted to differentiate only into cell types of the tissue from which they originate. However, many recent reports have shown that stem cells from one tissue can cross lineage boundaries to differentiate into cells of other lineages either *in vitro* or *in vivo* after transplantation. This plasticity, or ability for cells to trans-differentiate, has created great interest for their therapeutic potential in tissue engineering, particularly in the area of nerve regeneration. Thus adult tissue stem cells are likely to be of benefit for nerve regeneration due to both to their ability to trans-differentiate into neuronal and glial cells as well as through the secretion of a variety of growth factors and cytokines.

MSC can differentiate *in vitro* into neuronal cells expressing a variety of markers (Woodbury et al. 2000); (Khoo et al. 2008), (Krampera et al. 2007). However some research reports caution that the apparent differentiation of adult stem cells to a neuronal lineage could be an artefact due to toxicity of the chemical reagents (Lu et

al. 2004), (Krabbe et al. 2005). Nevertheless, other groups have provided functional electrophysiological data to support neural differentiation (Jang et al. 2010). Furthermore, the transplantation of neurally differentiated MSC has shown benefits for the treatment of various neurological deficits (reviewed in (Maltman et al. 2011). Targeted differentiation towards a glial cell phenotype has also been performed (Dezawa et al. 2001), (Caddick et al. 2006) and these cells have been used to treat both peripheral (Shimizu et al. 2007), (di Summa et al. 2011; di Summa et al. 2010) and spinal cord (Kamada et al. 2010) nerve injuries. The regenerative function of the stem cells is likely mediated by their secretion of a wide variety of neurotrophic and angiogenic factors such as NGF, BDNF, VEGF and angiopoietin (Caplan and Dennis 2006), (Munoz et al. 2005). Nevertheless, it has been repeatedly demonstrated that both native and differentiated MSC can prevent secondary degeneration, reduce cavity formation and stimulate axonal regeneration and remyelination in different spinal cord injury models (Kamada et al. 2010); (Someya et al. 2008); (Osaka et al. 2010); (Park et al. 2010); (Hejcl et al. 2010); (Sasaki et al. 2009); (Abrams et al. 2009); (Zurita et al. 2008). Moreover, MSC have been found to enhance angiogenesis in the ischemic boundary zone, stimulate neurogenesis, reduce the infarct size, restore the cerebral blood flow and blood brain barrier, and support neurological functional recovery after traumatic brain injury and stroke in adult rats (Brass 2006; English et al. 2006; Li and Chopp 2009; Chopp et al. 2009). In addition, many other soluble factors produced by MSCs contribute to their unique property to act as potent immunosuppressors and have immunoregulatory effects on a variety of cell types responsible for both adaptive and innate immunities (reviewed in (Hoogduijn et al. 2010), (Marigo and Dazzi 2011).

Clinical considerations for cell therapy

Based on experimental data cell based therapies have become a promising future treatment alternative for nerve injuries in recent years. In order to achieve the optimal treatment for nerve injuries one has to consider certain options such as donor age, senescence, number of cells and their abilities to proliferate and differentiate. From a clinical point of view the handling of the cells when transplanted in the injured zone has to be efficient as well. One of the problems is that culture protocols are different between laboratories and this has resulted in different gene expression patterns reported (Fehrer and Lepperdinger 2005). Cells cultured for prolonged periods of time begin to show morphological changes (Wagner et al. 2010). The senescence might be influenced by time in culture and in some studies also the age of the patient is an important factor (Wagner et al. 2009), (Stenderup et al. 2003). Telomerase shortening is an indication of aging and it gives a good track of mitotic events but the gene expression is also affected (Wagner et al. 2008). Clinically it is of importance that sufficient number of cells can be prepared in a reasonable period of time and that the cells keep their specific phenotype and properties.

AIMS OF THE STUDY

The overall goal of this thesis was to characterize human and rat bone marrow-derived mesenchymal stem cells and to evaluate their neuroprotective and growth-promoting effects after peripheral nerve and spinal cord injury.

The specific aims of the study were:

- To identify the phenotypic, molecular and functional characteristics of human and rat mesenchymal stromal cells differentiated into cells with a Schwann cell-like phenotype (Papers I & IV).
- To assess the effect of donor age and time in culture on capacity of mesenchymal stromal cells to secrete neurotrophic factors and support axonal growth *in vitro* (Paper II).
- To study the growth-promoting effects of a fibrin glue conduit seeded with human mesenchymal stromal cells after peripheral nerve injury and repair in adult rats (Paper III).
- To investigate the growth-promoting and neuroprotective effects of rat mesenchymal stromal cells after transplantation into the injured rat spinal cord (Paper IV).

MATERIALS AND METHODS

Experimental animals

Animal experiments were performed on adult female Fischer F344 rats (Paper III; 10-12 weeks old, average weight of 170g, Scanbur BK AB, Sweden) and female Sprague-Dawley rats (Paper IV; Taconic Europe A/S, Denmark). The animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were also approved by the Northern Swedish Committee for Ethics in Animal Experiments (2010-11-17; Um dnr A 127-10). All surgical procedures were performed under general anesthesia using a mixture of ketamine (Ketalar®, Parke-Davis; 100 mg/kg i.v.) and xylazine (Rompun®, Bayer; 10 mg/kg i.v.).

Culture of bone marrow-derived MSC

Samples of human bone marrow were obtained from the iliac crests of healthy donors during reconstructive surgery with informed consent. Procedures were approved by the Local Ethical Committee for Clinical Research in Umeå University (2003-12-23; Um dnr 03-425). Samples of rat bone marrow were harvested from the tibia and femur of adult female rats. A modification of a previously described protocol (Azizi et al. 1998) was used to isolate and to prepare primary cultures of human (Papers I-III) and rat (Paper IV) mesenchymal stem cells. Briefly, human bone marrow samples were rinsed thoroughly with alpha Minimum Essential Medium (α MEM) containing 10% (v/v) foetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (all Invitrogen Life Technologies, Paisley, UK or Täby, Sweden). The tibia and femur of rats were washed with cold normal saline and stored on ice in α MEM containing 20% FBS, penicillin 100 U/ml and streptomycin 100 μ g/ml. The epiphyses were removed and the bone marrow flushed with the medium using a 21G needle attached to 5 ml syringe. The cell suspension was centrifuged at 600-1500 g for 5 min and the cell pellet was filtered through a 70 μ m nylon mesh (BD Falcon, Becton, Dickinson and Company, Oxford, UK), plated in 75 cm² tissue culture flasks (Corning or Nunc, USA) and incubated at 37°C, 5% (v/v) CO₂. The isolation of MSC was based upon their ability to adhere to plastic surfaces (Azizi et al. 1998; Hofstetter et al. 2002). After 12-24 h in culture, the supernatant containing non-adherent cells was removed and discarded and fresh medium added. The cells attached to the culture flask were cultured at 37°C, 5% CO₂ for 2-3 weeks with medium changes every 48 h. When the cultures had reached 80% confluence, the cells were enzymatically detached from the flask using 0.25-1.25% trypsin/EDTA solution (Invitrogen Life Technologies) and re-seeded in new culture flasks at a density of 5 x 10³ cells per cm².

Differentiation of MSC into Schwann-like cells

The differentiation process for human MSC was initiated in sub-confluent cultures at the second and eleventh passage. Rat MSC were differentiated after passage 4. MSC were incubated with α MEM containing 1mM beta-mercaptoethanol (Sigma-Aldrich) for 24 hours. After washing with 0.1M phosphate-buffered saline (PBS, pH 7.4), medium was replaced with α MEM containing 10% FBS and 35 ng/ml all-trans-retinoic acid (Sigma-Aldrich) for 3 days. Human MSC were then incubated in growth medium supplemented with 5.7 μ g ml⁻¹ forskolin (MP Biomedicals,

Sweden), 10 ng ml⁻¹ basic fibroblast growth factor (Invitrogen), 5 ng ml⁻¹ platelet derived growth factor-AA (Millipore, Watford, UK) and 126 ng ml⁻¹ glial growth factor-2 (GGF-2, gift from Acorda Therapeutics Inc, USA). The cells were maintained in this supplemented medium for two weeks with medium changes every 72 h to establish differentiated cultures (Tohill et al. 2004;Caddick et al. 2006;Brohlin et al. 2009). Undifferentiated cells were cultured in parallel in order to maintain the same culture conditions. Cells were then trypsinised and analysed at passage 3 and 12.

Rat MSC were incubated in α MEM containing 10% FBS, 5 μ M forskolin, 10 ng/ml recombinant human basic-fibroblast growth factor (rh-bFGF; Invitrogen), 5 ng/ml recombinant human platelet derived growth factor-AA (rh-PDGF; Chemicon) and 200 ng/ml recombinant human heregulin-beta 1 (HRG; R&D Systems) for 7 days as described by (Dezawa et al. 2001).

Multipotency of the hMSC

To confirm the multipotency of the MSC, the cultures were differentiated into osteoblasts, chondrocytes or adipocytes according to the previously published protocols (Pittenger et al. 1999;Caddick et al. 2006;Mahay et al. 2008a). The cultures were treated for three weeks with the different induction media. Osteogenic induction medium comprised 10 mM β -glycerophosphate, 0.1 μ M dexamethasone and 100 μ g ml⁻¹ ascorbate (all Sigma-Aldrich) in α MEM (Invitrogen). Chondrogenic induction medium was prepared from 0.01nM dexamethasone, 50 μ g ml⁻¹ ascorbate, 40 μ g ml⁻¹ proline, 10ng ml⁻¹ transforming growth factor β 1 (all Sigma-Aldrich) and 1% ITS-Plus (BD Falcon) in high glucose Dulbecco's MEM (DMEM, Invitrogen). Adipogenic induction medium contained 1 μ M dexamethasone, 10 μ g ml⁻¹ insulin and 3-isobutyl-1-methylxantine (all Sigma-Aldrich) in high glucose DMEM. After the differentiation processes were complete, the cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and stained for osteoblasts, chondrocytes and adipocytes using Alizarin red, Toluidine Blue and Oil Red O, respectively (all Sigma-Aldrich).

Labeling of MSC for transplantation

Human MSC were labelled with fluorescent tracer PKH26 at passage 3 prior to transplantation into injured rat peripheral nerve (Paper III). Approximately 1×10^7 MSC were washed once with α MEM supplemented with 1% (v/v) penicillin-streptomycin (Invitrogen Täby, Sweden) and centrifuged at 1500 x g for 5 min, after which the supernatant was aspirated to leave no more than 25 μ l in the pellet. According to the manufacturer's recommendations, the cells were resuspended in 1 ml of diluent C solution (included in the PKH26 Red Fluorescent Cell Linker Kit, Sigma, USA) and then incubated in 4 μ M PKH26/1 ml diluent C dye solution for 5 min. The labelling reaction was stopped by adding 2 ml of FBS. Labelled cells were washed two times with α MEM/FBS by centrifugation for 10 min at 1500 g.

To express green fluorescent protein (GFP) in MSC prepared for transplantation into injured rat spinal cord (Paper IV), a retroviral expression system was used. The retroviral packaging cell line PT67 (Clontech) was propagated in high glucose DMEM (Invitrogen) supplemented with penicillin, 100 U/ml, streptomycin 100

$\mu\text{g/ml}$ (Invitrogen) and 10% FBS and transfected with a retroviral expression vector pLEGFPpuro. The GFP expression vector pLEGFPpuro resulted from substitution of the neomycin resistance gene in the retroviral vector pLEGFP-N1 (Clontech) by the puromycin resistance gene from pMSCVpuro (Clontech). The transfection of PT67 cells was done by a lipofection method with Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer. Successfully transfected cells were selected with puromycin and maintained (Clontech). For virus production, the packaging cells were seeded at confluence of about 60%, incubated overnight at 37°C and transferred into a 32°C incubator with 95% humidity and 5% CO_2 . After 72 hours, virus containing medium was collected and filtered through $0.45\ \mu\text{m}$ low-protein binding filter (Pall Corporation). For transduction, MSC at passage 4 were seeded at a density of 5×10^3 cells per cm^2 and 2 days later their growth medium was replaced with the viral medium containing $8\ \mu\text{l/ml}$ polybrene (Sigma-Aldrich). The cells were kept at 32°C for 24 h. After transduction, the viral medium was changed to a fresh MSC growth medium.

In addition, forty eight hours before transplantation MSC were supplemented with $10\ \mu\text{M}$ 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) to label the nuclei of dividing cells (Paper IV).

Cell proliferation assay

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Southampton, UK) was used for detection of cell proliferation. Briefly, human MSC cultures were trypsinized and seeded into 96-well culture plates (5×10^3 cells in $100\ \mu\text{l}$ of growth medium per well with 5 replicates per culture). After 2 hr, the old media was removed and $100\ \mu\text{l}$ of fresh media was added to each well and $20\ \mu\text{l}$ of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent, phenazine methosulphate solution per well was added and cultures were maintained at 37°C , 5% CO_2 for 2 h. The optical density of resulting formazan production was measured at $490\ \text{nm}$ using a Spectra Max 190 microplate reader (Molecular Device, Albertville, Minnesota, USA). Optical density measurements were taken every day at the same time point for three consecutive days.

Dorsal root ganglia co-culture

Dorsal root ganglia (DRG) were harvested from the spinal cords of adult male Sprague-Dawley rats using a previously described protocol (Mahay et al. 2008b; Caddick et al. 2006). Dissociated neurons were resuspended in modified Bottenstein and Sato's medium (F12 medium containing $100\ \mu\text{M}$ putrescine, $30\ \text{nM}$ sodium selenite, $20\ \text{nM}$ progesterone, $1\ \text{mg ml}^{-1}$ bovine serum albumin, $0.1\ \text{mg ml}^{-1}$ transferrin, $0.01\ \text{mM}$ cytosine arabinoside and $10\ \text{pM}$ insulin; all Sigma-Aldrich) or Neurobasal medium with B12 supplement (Invitrogen). The DRG neurons were seeded onto laminin-coated glass cover slips (Sigma-Aldrich) inserted into 6 well plates and incubated for 24 h (37°C , 5% CO_2). Twenty four hours prior to DRG harvest, cultures of undifferentiated MSC and differentiated MSC from the three patients were seeded onto $1.0\ \mu\text{m}$ pore size polyethylene terephthalate membrane cell culture inserts (BD Falcon) at a density of $150,000$ cells in $2\text{ml}/\text{insert}$ and incubated for 48hr (37°C , 5% CO_2). The inserts were checked for cell adherence

then placed into wells containing DRG neurons to establish the co-culture; these were allowed to incubate for 24 h (37°C, 5% CO₂). Additional controls with the cell-free inserts containing stem cell medium only were also analyzed.

Conduit preparation

Tubular fibrin conduit was molded from two compound fibrin glue (Tisseel® Duo Quick, Baxter SA, Switzerland). Fibrin glue contains; 70-110mg/ml fibrinogen, 2-9mg/ml of plasma fibronectin, 10-50 U/ml of factorXIII, 40-120 ug/ml of plasminogen, 3000 KIU/ml of aprotinin solution, 5 IU/ml of thrombin and 40mmol/l of calcium chloride. All components were mixed in sterile conditions according to manufacturer recommendations. A silicone mold with a centrally placed metal rod was used to prepare tubular 14-mm-long conduits with uniform 1-mm-thick walls and 2mm lumen (Kalbermatten et al. 2008). After glue polymerization, the rods and silicone mold were removed and fibrin glue conduits were stored in sterile Dulbecco's Minimum Eagle's Medium at room temperature.

Preparation of matrix

Fibrin matrix was produced by modifying a two compound fibrin glue (Tisseel® Duo Quick, Baxter SA, Switzerland) containing 70-110mg/ml fibrinogen, 2-9mg/ml of plasma fibronectin, 10-50 U/ml of factorXIII, 40-120 ug/ml of plasminogen, 3000 KIU/ml of aprotinin solution, 5 IU/ml of thrombin and 40mmol/l of calcium chloride. Both components of fibrin glue were diluted according to (Bensaid et al. 2003) et al. To dilute the fibrinogen 1:5 a buffer consisting of 10 ml of de-ionised water with 73.5 mg of sodium citrate, 16.9 mg sodium chloride, 249.9 mg glycine, 30 000 KIU aprotinin (Sigma, A-3428, Sigma-Aldrich, USA) and 150 mg albumin serum (Sigma, A-3428, Sigma-Aldrich, USA). For dilution of thrombin, a solution of 10 ml of de-ionised water with 58.8 mg calcium chloride, 87.1 mg sodium chloride, 30.3 mg glycine and 500 mg serum albumin was prepared and diluted 1:5. The two solutions were then combined as explained below producing a relatively soft fibrin clot (18/100) as per Bensaid et al (Bensaid et al. 2003) with 18 mg of fibrinogen per ml and 100 IU of thrombin per ml generating the optimal solution of 1/100 dilution of thrombin and 1/10 dilution of fibrinogen.

Incorporation of human MSC into the matrix

Fluorescently labeled cells were resuspended with diluted fibrinogen solution to a concentration of 80×10^6 per ml (Mosahebi et al. 2001). The diluted thrombin solution (25ul) was then injected into the lumen of a conduit and immediately an equal volume of cell/fibrinogen suspension was added. The matrix was allowed to polymerise for 10 minutes prior to surgical transplantation into the sciatic nerve defect. Conduits without cells were made as control.

Peripheral nerve injury and human MSC transplantation

A three cm skin incision was made over the left gluteal area. Under an operating microscope (Zeiss, Carl Zeiss, Germany), the sciatic nerve was exposed through splitting of gluteal and biceps muscle and then divided 5mm below the exit point from sciatic notch and 7 mm of sciatic nerve distal to the division was removed creating 10mm gap. The 14 mm long fibrin conduit was inserted in the gap, allowing for intubation of the nerve end 2 mms into the conduit, resulting again in

10 mm gap between proximal and distal sciatic stump. The conduit was fixed to the epineurium with one 10.0 Ethilon suture at each end. Tension was avoided and atraumatic handling and correct rotational alignment were employed throughout all procedures. The wound was then closed in layers. The animals were randomly divided into four experimental groups: (i) fibrin conduit filled with fibrin matrix, (ii) fibrin conduit filled with fibrin matrix + daily injections of cyclosporine A (Sandimmune, Novartis), (iii) fibrin conduit with fibrin matrix containing MSC and (iv) fibrin conduit with fibrin matrix containing MSC + daily injections of cyclosporine A. Cyclosporine A (Sandimmune, Novartis) was injected intraperitoneally at 1.5 mg per 100 g body weight starting from 24 hours before surgery. At week 2 the weight of animals was measure once again to ensure consistent delivery of the same concentration of cyclosporine. Operated animals were allowed to survive for 3 weeks to assess early regeneration events.

Spinal cord injury and rat MSC transplantation

After cervical laminectomy, the lateral funiculus and adjacent gray matter of the C4 spinal cord segment were transected on the left side. The rats were randomly divided into three experimental groups: (i) spinal cord injury without treatment (SCI, n=27), (ii) SCI followed by transplantation of undifferentiated MSC (n=23) and (iii) SCI followed by transplantation of differentiated MSC (n=36). Cells were transplanted within 30 minutes after SCI. Nine normal rats (immunohistochemistry and Western blotting), 5 rats at 1 week after Fast Blue application to the lumbar spinal cord and 5 rats at 1 week after anterograde labeling of rubrospinal axons with biotinylated dextran amine served as baseline controls (see below).

For transplantation, the cells were detached with Trypsin/EDTA, washed and concentrated to 75×10^3 cells/ μ l in α MEM without serum. The cells were transplanted at passage 5. After transfer into a siliconized glass micropipette (outer diameter 100 μ m) attached to a 5 μ l Hamilton syringe, 1.5-1.6 μ l of the cell suspension (100,000-120,000 cells) was slowly (10 minutes) pressure-injected into the lateral funiculus (depths 1.0 mm) at approximately 1 mm rostral and 1 mm caudal to the lesion site using Stoelting's Lab Standard Stereotaxic Instrument (Stoelting Co.). The micropipette was left in place for additional 2-3 minutes. Dura mater was covered with stretched parafilm and Spongostan®, muscles and skin were closed in layers, and the rats were given analgesic Finadyne (Schering-Plough, Denmark; 2.5 mg/kg, i.m.), saline (2 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.).

Retrograde labeling of rubrospinal neurons

In the experiments studying neuronal survival (n=18), rubrospinal neurons projecting to lumbar spinal segments were labeled with non-toxic retrograde fluorescent tracer Fast Blue (FB, EMS-Chemie GmbH, Germany) one week before cervical C4 spinal cord injury (Novikov et al. 2002; Novikova et al. 2002). Following a laminectomy, the L1 spinal cord segment was exposed and the dorsal portion of the left lateral funiculus including the rubrospinal tract (Paxinos 1985) was transected with fine scissors under an operating microscope. A small pellet prepared from 1-2 μ l of a 2% aqueous solution of the tracer Fast Blue was

placed into the lesion cavity and covered with a thin sheet of parafilm and a small piece of Spongostan®.

Anterograde tracing of rubrospinal axons

At 12 weeks after the first operation, the rats (n=15) were mounted in a stereotaxic frame and a 2 mm hole was drilled in the skull to allow access to the red nucleus. A glass micropipette (outer tip diameter 40-50 μm) filled with a 10% solution of biotinylated dextran amine in saline (BDA; 10 000 M_w , lysine fixable, Molecular Probes, Invitrogen) was inserted used into the magnocellular and parvocellular regions of the red nucleus (6.6 and 6.1 mm caudal to the bregma, 0.7 mm lateral to the midline, and 7.2 mm ventral to the bregma) using Stoelting's Lab Standard Stereotaxic Instrument (Stoelting Co.). BDA was injected iontophoretically by passing anodal current pulses of 10 μA (7 sec on/7 sec off) through the microelectrode for 10 minutes and the microelectrode was then left in place for additional 2-3 minutes (Novikov 2001) ; (Novikova et al. 2002). The rats were killed 1 week after labeling.

Tissue processing

For immunocytochemistry and analysis of DRG co-cultures, cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH = 7.4) at room temperature for 20 minutes. The animals were deeply anaesthetized with an intraperitoneal overdose of sodium pentobarbital and transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). In the experiments using anterograde BDA-labeling, the fixation consisted of a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4).

Peripheral nerve and conduit: The conduits were harvested with 2 mm of the distal nerve stump, postfixed in the same fixative for 2 h, cryoprotected in 10% and 20% sucrose for 48 hours and frozen in liquid isopentane. Serial longitudinal 16- μm -thick sections were cut on a cryomicrotome, thaw-mounted in pairs onto SuperFrost® Plus slides, dried overnight at room temperature and stored at -85°C before processing.

Brain stem and spinal cord: Spinal cord segments C2 and C3-C5 and the brain stem were harvested. For Western blotting, C3 spinal cord segments rostral to the injury site were divided into two halves in sagittal plane and immediately frozen in liquid nitrogen. For immunohistochemistry, spinal cord segments were post-fixed for 2-3 hours, cryoprotected in 10% and 20% sucrose for 2-3 days and frozen in liquid isopentane. Serial longitudinal 16- μm -thick sections were cut on a cryomicrotome (Leica Instruments), thaw-mounted in pairs onto SuperFrost®Plus slides, dried overnight at room temperature and stored at -85°C before processing. For fluorescence microscopy and cell counts, brainstems were postfixed for 18 hours at room temperature and 50- μm -thick serial transverse sections from the midbrain containing the red nucleus were cut on a vibratome (Leica Instruments), mounted on gelatin-coated glass slides, air dried, shortly immersed in xylene and coverslipped in DPX (Kebo Lab AB, Sweden). For demonstration of anterogradely BDA-labeled rubrospinal axons and arborizations, serial 50- μm -thick transverse sections (C2 spinal cord segments) and longitudinal sections (C3-C5 spinal cord segments) were

cut on a cryomicrotome or vibratome and processed according to a modified ABC method (Novikov 2001). Briefly, free-floating sections were washed in PBS, incubated for 6 hours at room temperature with avidin-biotin-peroxidase complex (1:1:100; Vector Laboratories) in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin, developed in a solution containing 0.05% of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich), 0.06% NiCl₂ and 0.003% H₂O₂, mounted on glass slides, counterstained with cresyl violet and cover slipped in DPX.

Immunohistochemistry

Immunostaining was performed on longitudinal 16- μ m-thick spinal cord and peripheral nerve sections and cells cultured on Lab-Tek® slides. After blocking with normal serum, the primary antibodies (see Table 1) were applied for 2 hours at room temperature. After rinsing in PBS, secondary goat anti-mouse and goat anti-rabbit antibodies Alexa Fluor® 488 and Alexa Fluor® 568 (1:300; Molecular Probes, Invitrogen) were applied for 1 h at room temperature in the dark. The slides were coverslipped with Vectashield® mounting medium containing DAPI (Vector Laboratories) or Prolong with DAPI (Invitrogen). The staining specificity was tested by omission of primary antibodies.

Western blotting

Cell cultures (Paper I) and C3 spinal cord segments (Paper IV) were homogenized in lysis buffer containing 5mM EGTA, 100mM PIPES, 5mM MgCl₂, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich) and then protein levels were determined using the D_c assay (BioRad). For the whole cell lysates, proteins were separated by denaturing (SDS) electrophoresis through 10% (w/v) acrylamide gels at 120 V using the Bio-Rad mini-Protean 3 (Bio-Rad laboratories). The separated proteins were transferred onto nitrocellulose membranes using the Biorad transblot system (Bio-Rad laboratories) (30 V, 90 min). For spinal cord tissue, 18 μ g protein was loaded per lane onto 10% (v/v) or 15% (v/v) SDS-polyacrylamide gels and resolved at 200V. Following electrophoresis, protein was transferred to nitrocellulose membranes (80V for 75 min) and then blocked in 5% (w/v) non-fat milk in Tris buffered saline with Tween (TBS-T) for 1h. The primary antibodies (see Table 1) were diluted in the blocking solution and incubated with membranes overnight at 4°C. Additionally, beta tubulin antibody (rabbit polyclonal; 1:2000; Abcam, ab6046, Cambridge, UK) was used as a loading control. After 6 x 5 min washes in TBS-T, rabbit IgG HRP-linked secondary antibody (1:1000; Cell Signaling Technology) was applied for 1 h at room temperature. Finally the membranes were washed 6 x 5 min in TBS-T and the blots exposed to ECL reagent (Amersham Biosciences, UK and GE Healthcare) and developed onto Kodak XPS films. To ensure equal protein loading of samples, the membranes were stripped of antibody using 100 mM glycine, pH 2.9, and then processed for blotting with mouse anti-actin (1:5000; Millipore). Films were scanned using an Epson Photoscanner and then 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 actin or beta tubulin.

Table 1. Primary antibodies for immunohistochemistry and Western blotting

Antibody	Dilution	Source	Method	Paper
Mouse anti-CD14	1:1000	Millipore	IHH	I
Mouse anti-CD45	1:200	Millipore	IHH	I
Mouse anti-CD54	1:75	Millipore	IHH	I
Mouse anti-CD90	1:50	Serotec	IHH	I
Mouse anti-Stro-1	1:50	R&d Systems	IHH	I
Rabbit anti-p75	1:500	Promega	IHH	I
Mouse anti- β III tubulin	1:500 - 1:1000	Sigma-Aldrich	IHH	I, II, III
Mouse anti-ED1	1:100	Abcam	IHH	III
Mouse anti-vimentin	1:1000	Chemicon	IHH	IV
Mouse anti-laminin	1:200	Chemicon	IHH	IV
Mouse anti-fibronectin	1:200	Chemicon	IHH	IV
Mouse anti-nestin	1:2000	Chemicon	IHH	IV
Rabbit anti-GFAP	1:500	Dakopatts	IHH	IV
Rabbit anti-GFP	1:500	Invitrogen	IHH	IV
Rabbit anti-brdu	1:2000	Sigma-Aldrich	IHH	IV
Rabbit anti-serotonin	1:1000	Sigma-Aldrich	IHH	IV
Rabbit anti-CGRP	1:1000	Chemicon	IHH	IV
Mouse anti-OX42	1:250	Serotec	IHH	IV
Mouse anti-neurofilament proteins 68 kda, 160 kda and 200 kda	1:200	Zymed Laboratories	IHH	IV
Rabbit anti-S100	1:500	Dakopatts	IHH, WB	I, IV
Mouse anti-GFAP	1:200	Stratech Scientific	WB	I
Rabbit anti-p75	1:300	Promega	WB	I
Mouse anti-erbb3	1:200	Santa Cruz	WB	I
Rabbit anti- β tubulin	1:2000	Abcam	WB	I
Rabbit anti-BDNF	1:200	Santa Cruz	WB	IV
Rabbit anti-NT3	1:200	Santa Cruz	WB	IV
Rabbit anti-VEGF	1:200	Santa Cruz	WB	IV
Rabbit anti-laminin	1:500	Sigma-Aldrich	WB	IV
Mouse anti-actin	1:5000	Millipore	WB	IV

Fluorescence activated cell sorting (FACS)

The MSC were additionally identified by flow-cytometry using anti-human CD44 antibodies (Serotec). After detachment from the culture flasks, the cells were re-suspended in FACS buffer (PBS, pH 7.4 with 3% (v/v) FBS and 0.01% (w/v) sodium azide) and incubated with phycoerythrin (PE)-conjugated antibodies in the dark for 20 min on ice. Cells were washed once and re-suspended in a small volume

of FACS buffer to achieve a concentration of $2\text{-}5 \times 10^6$ cells ml^{-1} . The CD44⁺ PE-labelled cells were detected using a FACSCalibur (DiVa, BD Biosciences, San Jose, USA) and analyzed with CellQuest Pro software.

RT-PCR for glial markers and neurotrophic factors

The RNeasyTM mini kit (QIAGEN Ltd., UK) was used for the isolation of total RNA from the cell pellets of the undifferentiated MSC and differentiated MSC from the three patients as previously described (Mahay et al. 2008a). Primers for glial markers GFAP, erbB3, p75 and S100 were synthesised by Invitrogen and for neurotrophic factors BDNF and NT-3 by Sigma (Table 2). The optimum annealing temperature for each primer pair was determined experimentally. The One-Step RT-PCR kit (QIAGEN Ltd.) was used for all RT-PCRs as per the manufacturer's instructions with the addition of 1 ng total cellular RNA or RNase free water (negative control).

For *glial markers*, an MJ Research PTC-200 thermal cycler was used for all reactions. The cycling parameters were as follows: a reverse transcription step (50 °C, 30 min), a denaturation/reverse transcriptase inactivation step (95 °C, 15 min) followed by 35 cycles of denaturation (95 °C, 30 sec), annealing (30 sec, see Table 2) and primer extension (72 °C, 1 min) followed by final extension incubation (72 °C, 5 min). Once the thermal cycling was complete, a qualitative assessment of the resulting amplicons was done by electrophoresis (50V, 90min) through a 2% (w/v) agarose gel (Melford Laboratories Ltd, UK) followed by staining with ethidium bromide (0.5 mg ml^{-1} , Sigma-Aldrich). The length (in base pairs) of the PCR products was estimated by comparison with DNA standards, Hyperladder IV (Bioline, UK). Images were captured using an AlphaImager 2200 (AlphaInnotech, USA) gel documentation system. The RT-PCR procedure was repeated (n = 5) for each of the six groups (MSC and differentiated MSC for P1, P2 and P3 patients). The nucleotide sequence of each amplicon was confirmed using the Big DyeTM Terminator sequencing kit (Applied BioSystems Incorporated, USA) and protocol followed by sequence analysis on the Prism 3100 Genetic Analyzer (Applied Biosystems Incorporated). It was necessary to use biphasic PCR methodology to detect the low-level transcripts of GFAP and erbB3. Following RT-PCR amplification (first round) of the GFAP and erbB3 transcripts, the reaction products were purified using a QiaQuick PCR clean-up (QIAGEN) and re-amplified using a standard PCR kit (Bioline, UK). The resulting products were subjected to agarose gel electrophoresis and DNA sequencing to verify their identity.

For *neurotrophic factors*, a thermocycler (Biometra, Göttingen, Germany) was used with the following parameters: a reverse transcription step (50 °C, 30min), a nucleic acid denaturation/reverse transcriptase inactivation step (95 °C, 15min) followed by cycles of denaturation (95 °C, 30sec) and annealing (30sec, optimised per primer set as described in Table 2) and primer extension (72 °C, 1min) followed by final extension incubation (72 °C, 5min). PCR amplicons were electrophoresed (50V, 90min) through a 1.5% (w/v) agarose gel and the size of the PCR products estimated using Hyperladder IV (Bioline, UK). Samples were visualised under UV illumination following GelRedTM nucleic acid stain (BioNuclear, Sweden) incorporation into the agarose.

Table 2. Forward and reverse primer (all 5'→3') pairs with annealing temperatures

Primers	Forward and reverse primer	Annealing temperature	Paper
GFAP	GTC CAT GTG GAG CTT GAC G and CAT TGA GCA GGT CCT GGT AC	60.7°C	I
erbB3	GGA GTC TTG CCA GGA GTC T and AGG AGT CAG CAG ACT GTG G	54.0°C	I
P75	TGG ACA GCG TGA CGT TCT CC and GAT CTC CTC GCA CTC GGC GT	60.7°C	I
S100	GGA AAT CAA AGA GCA GGA GGT and ATT AGC TAC AAC ACG GCT GGA	54.8°C	I
NGF	ATACAGGCGGAACCACTCAG and GTCCACAGTAATGTTGCGGGTC	63.7°C	II
GDNF	CACCAGATAAACAAATGGCAGTGC and CGACAGGTCATCATCAAAGGCG	55.6°C	II
VEGF	TACCTCCACCATGCCAAGT and TGCATTACATTTGTTGTGC	53.5°C	II
GAPDH	GAAGGTGAAGGTCGGAGT and CAAGCTTCCCGTTCTCAGC	62.0°C	II
BDNF	AGAGGCTTGACATCATTGGCTG and CAAAGGCACTTGACTACTGAGCATC	63.7°C	II
BDNF	ATGGGACTCTGGAGAGCGTGAA and CGCCAGCCAATTCTCTTTTTGC	66.5°C	IV
NT-3	GGGAGATCAAAACGGGCAAC and ACAAGGCACACACACAGGAC	61.6°C	II
NT-3	CTTATCTCCGTGGCATCCAAGG and TCTGAAGTCAGTGCTCGGACGT	65.5°C	IV
β-actin	ACTATCGGCAATGAGCGGTTC and AGAGCCACCAATCCACACAGA	65.0°C	IV

Enzyme-linked immunosorbant assay (ELISA)

MSC were seeded at an identical density to those used for neurite outgrowth analysis (15000 cells per 200µl media) and maintained for 48-72h. The supernatant from the cells was then collected and analysed by ELISA using the ChemiKine™ BDNF sandwich ELISA kits (Chemicon, UK) or VEGF sandwich ELISA kit (RayBio, GA, USA) according to the manufacturer's protocol. All samples were analysed in triplicate and the absorbance was measured at 450 nm (Spectra Max 190 microplate reader (Molecular Device, Albertville, MN, USA)).

Morphological analysis

Analysis of MSC markers: Expression of MSC markers was quantified in 10 randomly selected areas of the LabTek slide at x250 final magnification using 400x400 μ m sampling frame.

Neurite outgrowth analysis: The images from slides containing adult rat DRG neurons were captured for quantification at x10 magnification using an Evolution QEi monochrome digital camera (Media Cybernetics, USA) or Nikon DXM1200 digital camera. In Paper I, neurite outgrowth was assessed using three independent parameters: percentage of process-bearing neurons, length of longest neurite and total neurite density (total no. of intersection points of a concentric circle set with radii increasing by 33 μ m) using the previously described SigmaScan Pro 5 software macro (Caddick et al. 2006; Mahay et al. 2008b). Four independent co-culture experiments were carried out and neurite outgrowth assessed. In Paper II, image analysis was performed using NeuroLucida software (MBF Bioscience, USA). All neurites were traced to generate total neurite outgrowth data (in μ m). Three independent co-culture experiments were carried out for neurite outgrowth assessment.

Analysis of fibrin conduit and peripheral nerve: Preparations were photographed with a Nikon DXM1200 digital camera attached to a Leitz Aristoplan microscope. Longitudinal sections of the conduits immunostained with β III tubulin antibody were photographed at 250x magnification at 3 mm distal to the entry point into the conduit to analyze the density of axonal regeneration. Images were processed with help of Adobe Photoshop CS3, pasted onto a single canvas and trimmed to reconstruct a single image of a band of a standard width perpendicular to the long axis of the conduit or the nerve graft. For analysis of macrophage activation (ED1-immunostaining), pictures were taken from the central part of the conduit at x400 magnification. One or more photographs were taken depending on the width of the nerve in the particular specimen. Area of staining (β III tubulin and ED1) was calculated after image calibration using Image Pro Plus software (Media Cybernetics, Bethesda, USA). Distance of regeneration was measured with a calibrated graticule of a Leitz microscope to the nearest 0.1 mm on longitudinal sections of the conduits from the entry point of the conduit to the furthest axon stained against β III tubulin and expressed in micrometers. Distance of regeneration was measured on 3 consecutive sections from each conduit spaced at 50 micrometers apart.

Quantification of axonal sprouting and glial reaction in spinal cord: Serotonin-positive nerve fibers in the ventral horn, CGRP-positive nerve fibers in Rexed's lamina III, GFAP-positive astrocytes and OX42-positive microglial cells in lamina VII were studied in 20 randomly selected transverse sections from the C2 spinal cord segments of normal rats and at 6 weeks after SCI and MSC transplantation. Images were captured at 400 final magnifications with a Nikon DS-U2 digital camera and imported into Image-Pro Plus software (Media Cybernetics, Inc., USA). The resolution was 3840x3072 pixels for images of nerve fibers and 1280x1024 pixels for images of glial cells. The relative tissue area occupied by labeled profiles

was quantified in 100x100 μ m area for serotonin-positive axons, 50x150 μ m area for CGRP-positive axons and in 150x150 μ m area for glial cells.

Counts of rubrospinal neurons and axons: The nuclear profiles of the retrogradely labeled neurons were counted in all sections through the red nucleus at 250x magnification. The total number of profiles were not corrected for split nuclei since the nuclear diameters were small in comparison with the section thickness used (Novikova et al. 2000;Novikov et al. 2002;Novikova et al. 2002). We have previously demonstrated that the accuracy of this counting technique in estimation of retrograde neuronal cell death is similar to that obtained with the physical disector method (Ma et al. 2001) and counts of neurons reconstructed from serial sections (Novikov et al. 1997).

BDA-labeled rubrospinal axons were studied in 25 randomly selected transverse sections from the C2 spinal cord segments of normal rats and at 13 weeks after SCI and dMSC transplantation as described previously (Novikova et al. 2002). In brief, rubrospinal axons in dorso-lateral funiculus and terminal arborizations in lamina V were captured in four non-overlapping areas at 2,000 final magnification with a Nikon DXM1200 digital camera and imported into Matrox Inspector 3.1 software (Matrox Electronic Systems Ltd., Canada). The final image size was 1280 x 1024 pixels and corresponded to the area of 54.18 x 43.35 μ m. The density of labeled profiles was quantified for each image at a constant discrimination level and an average value was calculated for each section.

Image processing

Preparations were photographed with a Nikon DXM1200 or Nikon DS-U2 digital cameras. The captured images were resized, grouped into a single canvas and labeled using Adobe Photoshop CS3 or CS4 software. The contrast and brightness were adjusted to provide optimal clarity.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (GraphPad Software Inc., USA). The data are expressed as mean \pm standard error of the mean (SEM) following the *post hoc* Newman-Keuls Multiple Comparison Test (Papers I and IV) or Bonferonni test (Paper III). The data for neurite outgrowth quantification are expressed as mean \pm SEM following one-way ANOVA followed by Bonferonni's Multiple Comparison Test (Paper I) or Newman-Keuls Multiple Comparison Test (Paper II).

RESULTS

Characterisation of human MSC (Paper I)

MSC markers: Cultured undifferentiated human MSC readily adhered to plastic surfaces and the majority of the cells in primary cultures from all three patients displayed a flattened fibroblast-like morphology. During growth factor-induced differentiation, MSC changed their shape from flat to spindle-like morphology.

Immunocytochemical labelling of the MSC showed positive staining for stro-1 in 80% MSC of the three patients (Fig. 1). Staining for haemopoietic stem cell surface markers CD14 (Fig. 2A, E and I) and CD45 (Fig. 2B, F and J) was negative in the MSC of all three patients (P1, P2 and P3). More than 80% of MSC were positive for MSC surface markers CD54 (Fig. 2C, G and K) and CD90 (Fig. 2D, H and L). Flow-cytometry showed that approximately 40% of the MSC from each of the three patients expressed CD44. In a 2×10^6 aliquot (1 ml) of cells from each patient, 8.2×10^5 P1 cells were CD44+ cells, 8.0×10^5 P2 cells were CD44+ and 8.4×10^5 P3 cells were CD44+ (Fig. 3).

Multilineage potential: The multipotency of MSC from the three patients, P1 (Fig. 4A-C), P2 (Fig. 4D-F) and P3 (Fig. 4G-I), was demonstrated by their ability to differentiate into chondrocytes, osteoblasts and adipocytes in appropriate culture conditions. The sulphated proteoglycan of chondrocytes was stained blue with Toluidine Blue (Fig. 4A, D and G), areas of calcification around osteoblasts were labelled red with Alizarin red (Fig. 4B, E and H) and the lipid droplets in the adipocytes were stained red with Oil red (Fig. 4C, F and I).

Glial cell markers: The expression of the glial marker *proteins* was confirmed by immunocytochemical staining. S100 and low affinity NGF receptor p75 (Fig. 5) were detected in 80% of differentiated human MSC from the three patients compared to the MSC which showed negative staining for each patient as shown by the DAPI counterstaining.

The presence of the glial cell marker *gene transcripts* was detected by RT-PCR methodology (Fig. 6). This showed that differentiated MSC express the transcripts for S100 (408 bp amplicon), p75 (371 bp amplicon) and GFAP (406 bp amplicon) compared to the undifferentiated MSC from the three patients. The differentiated MSC expressed the transcripts for erbB3 (238 bp amplicon). In the differentiated MSC, the transcript levels for the genes of interest were similar in all three patients, except for the slightly lower levels of S100B and p75 transcripts in P2 and P1, respectively. RT-PCR amplification efficacy of the mRNA was confirmed by the amplification of the constitutively expressed GAPDH (226 bp amplicon) housekeeping gene.

Western blotting experiments (Fig. 7) showed that there was a higher level of *protein expression* for the glial proteins S100 (10 kDa), GFAP (51 kDa), p75 (75 kDa) and erbB3 (189 kDa) in dMSC compared to MSC. In the differentiated MSC groups, the protein levels were similar in all three patients, except for slightly

increased levels of GFAP in P1. The equivalence of total protein loading was confirmed using the constitutively expressed β III tubulin (37 kDa) as a control.

Characterization of rat MSC (Paper IV)

Although undifferentiated rat MSC in primary cultures displayed significant variability, the majority of cells had flat fibroblast-like morphology. Following treatment with induction medium (Dezawa et al. 2001), MSC slowly reached confluence and many cells changed their shape from flat to spindle-like morphology. In agreement with previous observations (Hofstetter et al. 2002; Caddick et al. 2006), all MSC were immunopositive for vimentin, laminin and fibronectin (Fig. 1A,B,C). A small proportion of MSC also expressed nestin ($6.1\% \pm 2.0\%$; Fig. 1D) and S100 protein ($6.5\% \pm 1.7\%$). Immunoreactivity for the S100 protein was found in $53\% \pm 2.0\%$ of differentiated MSC (Fig. 1E). In addition, differentiated MSC began to express glial marker GFAP ($< 1\%$; Fig. 1F).

Proliferation rates of human MSC (Papers I & II)

The proliferation rates of MSC and dMSC from the three patients increased with time over a 72 h period (Fig. 10; Paper I). As measured over a 72 h period, the differences in proliferation rates of the MSC and dMSC for all three patients were statistically significant ($p < 0.001$; Fig. 10; Paper I).

Culturing the undifferentiated MSC over a time limit for ten weeks (T2-T11) showed a progressively decreased proliferation rate for the old donors compared with the young donors. Cumulative population doublings (PD) showed a continual linear trend for young donor MSC whereas the old donor cells began to plateau after 5 passages (Fig 3B; Paper II).

Expression of neurotrophic factors (Papers II & IV)

The presence of the trophic factor gene transcripts in *human MSC* was detected by semi-qualitative RT-PCR methodology using GAPDH as a house-keeping control gene (Fig. 2A). Undifferentiated MSC (passage 3) from both young and old donors expressed transcripts for NGF, GDNF, NT-3, BDNF and VEGF, all molecules which have been reported to promote DRG neurite outgrowth. Differentiation of MSC had no effect on the levels of NGF or GDNF but resulted in the down-regulation of NT-3 transcript. In contrast, both BDNF and VEGF transcript levels were elevated by the differentiation process (Fig 2A). To confirm the gene expression changes led to enhanced translation and production of protein we performed ELISA on the cell supernatants. Both BDNF and VEGF were expressed to a higher extent in the dMSC compared with MSC ($p < 0.001$) in young and old donors.

In *rats MSC*, transcripts for BDNF but not NT-3 were detected in both undifferentiated and differentiated cells (Fig. 2A). RT-PCR amplification efficacy of the mRNA was confirmed by the amplification of the β -actin housekeeping gene. Semi-quantitatively, the results suggested that BDNF transcript levels were elevated in dMSC. Therefore, to confirm that differentiation of the MSC lead to increased protein expression, we analysed BDNF levels in the cell supernatants (Fig. 2B).

MSC produced 6.4 ± 0.3 pg/ml BDNF and this was significantly ($P < 0.001$) increased to 99.7 ± 1.8 pg/ml BDNF from the dMSC.

Effects of human MSC on neurite outgrowth (Papers I & II)

Immunocytochemical staining for β III tubulin showed extensive neurite outgrowth by DRG neurons co-cultured with MSC from the three patients, P1 (Fig. 8A and B; Paper I), P2 (Fig. 8C and D; Paper I) and P3 (Fig. 8E and F; Paper I). The neurite outgrowth by the DRG neurons was markedly enhanced in co-cultures with dMSC (Fig. 8B, D and F; Paper I) compared to the co-cultures with MSC (Fig. 8A, C and E; Paper I) from the three patients. These qualitative observations were confirmed by the quantification of three parameters of neurite growth: percentage DRG neurons sprouting neurites, length of longest neurite and neurite density. In all cases, the DRG co-cultured with dMSC showed a statistically significant ($p < 0.01$ or $p < 0.001$; Paper I) increase in neurite sprouting (Fig. 9A; Paper I), neurite length (Fig. 9B; Paper I) and total neurite density (Fig. 9C; Paper I) compared to the co-cultures with MSC.

It appeared that differentiated cells taken from *young* donors generated the most neurite outgrowth (Fig 1A; Paper II). These observations were confirmed by using quantitative computerized image analysis of total neurite length (Fig 1B; Paper II). Neither growth medium nor differentiation medium alone significantly affected total neurite length compared with DRG neurons grown alone without tissue culture inserts. Both young and old donor MSC significantly ($P < 0.001$ and $P < 0.05$ respectively) enhanced total neurite length. Differentiation of young donor MSC produced the greatest total neurite length ($8195 \pm 254.3\mu\text{m}$) and significantly enhanced the levels above the undifferentiated cells ($\text{MSC} = 6967 \pm 252.2\mu\text{m}$, $P < 0.01$). In contrast differentiated old donor MSC showed no significant difference from undifferentiated old donor MSC ($6492 \pm 295.7\mu\text{m}$ and $6898 \pm 292.3\mu\text{m}$ respectively).

The enhanced effect of differentiated cells from the young donors was also maintained at late passages ($\text{MSC} = 7295 \pm 456.1\mu\text{m}$, $\text{dMSC} = 9523 \pm 505\mu\text{m}$ total neurite length; $P < 0.001$).

Transplantation of human MSC into injured sciatic nerve (Paper III)

Analysis of conduits and transplanted cells: At 3 weeks post implantation in both groups which received daily cyclosporine injections the biodegradation of the conduit wall was already underway in contrast to groups without cyclosporine treatment. This difference was visible both micro- and macroscopically, with clear difference between the size of harvested conduits. In groups where the animals did not receive injections the conduits had the same width as conduits prior to transplantation while in groups where the animals received cyclosporine the conduits were uniformly narrow resembling the size of sciatic nerve. Analysis of tissue sections showed that PKH26 labeled cells were present within the remnants of conduits both in untreated and cyclosporine A treated groups (Fig 1).

Distance of axonal regeneration: The distance of regeneration was measured on the longitudinal sections of the conduits from the entry point of the conduit until furthest axon stained with β III tubulin and expressed in micrometers (Table 1). The survival time of 3 weeks was chosen to assess early regenerative events at the time when pioneering axons in the growth cone are crossing a 10mm gap as has been found in our previous experiments with hollow fibrin glue conduit (Kalbermatten et al. 2008). However, in animals receiving the same fibrin conduit but filled with fibrin matrix, regeneration distance was reduced to $2851 \pm 176 \mu\text{m}$. Treatment with transplantation of MSC without cyclosporine injections or cyclosporine alone had no significant effect on regeneration distance (Table 1). However, when transplantation of MSC was combined with cyclosporine treatment, the regenerating axons crossed the conduit, continued into the distal nerve stump and past the distal end of the harvested specimen (additional 2-3mm of tissue). Since the distance of axonal growth was greater than the length of the conduit, it was impossible to assess the actual distance of regeneration and the value of 12mm (the length of the entire conduit from the proximal entry point) was used in the Table 1.

Area occupied by regenerating axons: The area occupied by axons stained with β III tubulin at 3 mm from the proximal nerve stump inserted into the conduit was measured to evaluate the density of axonal regeneration (Table 1). The control value for fibrin conduit filled with fibrin matrix was $23822 \pm 2930 \mu\text{m}^2$. Treatment with cyclosporine significantly increased the area of axonal staining by 254% ($P < 0.001$; Table 1). Surprisingly, transplantation of MSC alone did not (Table 1). However, when MSC were combined with cyclosporine treatment, the area of staining was similar to cyclosporine treatment alone and significantly increased compared with the control group ($P < 0.001$, Table 1).

Area occupied by macrophage infiltration: The immediate inflammatory response was investigated by measuring an area of macrophage infiltration using immunolabeling with ED1 antibody. In rats receiving fibrin conduit filled with fibrin matrix, the area of ED1-staining was $21.5 \pm 2.5\%$ (Table 1). Treatment with cyclosporine did not affect macrophage reaction and transplantation of MSC increased macrophage infiltration to $41.1 \pm 3.4\%$ ($P < 0.001$; Table 1). However, combined MSC transplantation and cyclosporine injections decreased the area of ED1 staining to $9.9 \pm 1.7\%$ ($P < 0.05$; Table 1).

Transplantation of rat MSC into injured spinal cord (Paper IV)

Distribution of rat MSC after transplantation: The BrdU- and GFP-labeled MSC were found in the injection sites rostral and caudal to the spinal hemisection and in the trauma zone (Fig. 3). The number of labeled cells was decreased at 5 weeks post-operatively and only a few labeled MSC could be found at 8 weeks after implantation into the injured spinal cord. No significant migration from the corresponding injection sites was observed. Additional immunostaining with pan-neurofilament antibodies revealed that axons readily entered sites of cell transplantation and in many cases could be found in close association with grafted MSC (Fig. 3).

Transplantation of MSC and neurotrophic factors expression in the host tissue:

Western blot analysis of C3 spinal cord segments was performed to determine expression levels of various growth promoting molecules (Fig. 4). The 14 kDa mature form of BDNF was detected in control tissue and showed a significantly ($P<0.05$) reduced expression level in spinal cord hemisection treated animals. The addition of MSC or differentiated MSC did not alter expression levels of BDNF. The levels of NT-3 protein were not altered by spinal cord hemisection in the absence or presence of cells (Fig. 4). Vascular endothelial growth factor (VEGF) migrated in the SDS-polyacrylamide gels as a dimer of 42 kDa and a monomer of 21 kDa. The dimer expression levels were unaffected by spinal cord hemisection or treatment with cells. However, the monomer was not detected in control tissue and was expressed at low levels in spinal cord hemisection treated animals. The addition of differentiated MSC significantly ($P<0.01$) elevated the expression levels of VEGF monomer and there was also a smaller increase upon treatment with undifferentiated MSC (Fig. 4). Laminin expression was significantly ($P<0.05$) elevated in spinal cord hemisection treated animals compared with control animals and addition of cells did not further change the levels.

Effects of rat MSC on axonal regeneration and glial reaction: At 6-8 weeks after spinal cord hemisection, only few thin pan-neurofilament immunolabeled terminals were found within the trauma zone. Transplantation of MSC and differentiated MSC induced extensive ingrowth of neurofilament-positive fibers, serotonin-positive raphespinal axons and calcitonin gene-related peptide (CGRP) immunostained sensory axons into the trauma zone (Fig. 5). An interesting finding was also that in some experiments MSC promoted regeneration of raphespinal fibers across the lesion site and into the caudal spinal cord (Fig. 5E,F).

Quantification of axonal arborizations in the C2 spinal segment at 6 weeks postoperatively demonstrated that spinal cord hemisection induced significant sprouting of serotonin-positive raphespinal axons in the ventral horn but did not affect the number CGRP-labeled sensory terminals in the dorsal horn (Fig. 6A,B). Transplantation of differentiated MSC but not MSC attenuated sprouting of raphespinal axons ($P<0.05$; Fig. 6A). However, both MSC and differentiated MSC caused aberrant ingrowth of CGRP-immunoreactive axons into Rexed's lamina III ($P<0.01$; Fig. 6B).

Spinal cord hemisection increased immunoreactivity of GFAP-positive astrocytes and OX42-positive microglial cells in lamina VII rostral to the injury site. Transplantation of MSC significantly attenuated astroglial and microglial reactivity ($P<0.01$; Fig. 6C,D).

Effects of rat MSC on rubrospinal neurons: In order to assess possible neuroprotective effects of MSC, we pre-labeled rubrospinal neurons projecting to the lumbar spinal cord with the fluorescent dye Fast Blue. In control animals, at 1 week after Fast Blue application to the lumbar spinal cord, the red nucleus contained $1,704 \pm 58$ labeled neuronal profiles located in the ventral portions of the magnocellular and parvocellular regions of the nucleus. Spinal cord hemisection at C4 cervical level induced significant cell death in the red nucleus and at 8 weeks

postoperatively only about 50% of the Fast Blue-labeled rubrospinal neurons remained in the nucleus (Fig. 7A). Transplantation of MSC had no neuroprotective effect of rubrospinal neurons. In contrast, differentiated MSC promoted survival of rubrospinal neurons to 68% ($P < 0.05$; Fig. 7A) and increased the number of BDA-labeled rubrospinal axons in the dorso-lateral funiculus rostral to the injury site ($P < 0.01$, Fig. 7B and Fig. 8A,C,E). The density of labeled rubrospinal arborizations in Rexed's lamina V was not affected by differentiated MSC transplantation (Fig. 7C and Fig. 8B,D,F) and no regeneration of rubrospinal axons was found in the trauma zone.

DISCUSSION

Molecular and functional characteristics of human MSC (Paper I)

The MSC were isolated from three healthy human donors and their cellular identity was verified by their fibroblastic morphology and confirmation of the expression of MSC-specific cell surface markers, stro-1, CD44, CD54 and CD90 and negative expression for CD14 and CD45 (Zhou et al. 2005; Pittenger et al. 1999). The multilineage potential of these cells was further evidenced by their ability to undergo differentiation into a variety of cell types (Pittenger et al. 1999; Krampera et al. 2007).

The morphological changes were clearly evident within 4-5 days of culture in the presence of GGF-2. The induction process was similar to that previously observed in rat studies. After induction along a glial lineage for two weeks, the differentiated MSC displayed a bipolar, elongated spindle-shape, which is characteristic of SC spindle-like morphology.

In addition to the obvious morphological changes, differentiated MSC showed expression of glial cell markers. Importantly, this was true for the cells derived from three different patients. As with the rat MSC, the human MSC did not express any of the glial markers (Caddick et al. 2006). Specifically, we showed that differentiated human MSC express glial markers p75, GFAP, S100 and the GGF-2 receptor, erbB3 at both the transcriptional and translational level. Although we did not compare the glial marker expression profile of the differentiated human MSC with that of human SC in the present study, it is well known that human SC readily express the standard glial markers such as S100 and GFAP (Bianchini et al. 1992; Mosahebi et al. 2001; Gonzalez-Martinez et al. 2003). There is also no reason to believe that the expression profile of human SC is markedly different from that of rat SC.

Neurotrophic potential of MSC (Papers II & IV)

In Paper II we described mRNA transcripts for NGF, GDNF, NT-3, BDNF and VEGF expressed in undifferentiated human MSC. In a previous study, a screen of a human MSC cDNA library also revealed transcripts for BDNF and NGF but not NT-3 (Crigler et al. 2006). Interestingly, expression of BDNF and NGF proteins were restricted to sub-populations of human MSC indicating heterogeneity of the cultures which could account for our ability to detect NT-3 transcripts in our cultures. In contrast, rat MSC in Paper IV did not express NT-3.

When we treated the human MSC with the glial cell growth factors we found BDNF transcript expression was enhanced and a similar effect on BDNF protein levels was confirmed by ELISA. A similar effect was achieved with rat MSC in Paper IV. This result is consistent with previous studies in rat MSC (Mahay et al. 2008a). BDNF levels correlate with enhancement of SH-SY5Y (Crigler et al. 2006) and DRG (Mahay et al. 2008b) neurite outgrowth in response to MSC. However, our study showed that BDNF levels were increased in both young and old donor human MSCs treated with glial growth factors, whereas neurite outgrowth was only further enhanced by young donor MSC treated in this manner. This likely suggests that

some other growth factors have an overriding effect on differentiated MSC evoked neurite outgrowth.

Expression of VEGF in MSC has been extensively documented from the viewpoint of the angiogenic capacity of the cells. However, more recently it was shown that MSC treated with a similar mix of glial growth factors as used in our study, could increase levels of VEGF, which was directly correlated with neurite outgrowth (Park et al. 2010). VEGF blocking antibodies also reduced the positive effect of MSC on axonal outgrowth of spinal nerves in a demyelinating organotypic spinal cord slice culture (Park et al. 2010). VEGF secreted by adipose derived stem cells can also protect cultured neurons against glutamate evoked excitotoxicity (Lu et al. 2010). We found that VEGF was up-regulated by the differentiation process, but similar with BDNF, this response was also observed in old donor MSCs. In Paper IV we demonstrated that after transplantation, MSC changed the expression of VEGF in the injured spinal cord tissue rostral to the injury but do not affect expression of neurotrophins BDNF and NT-3.

Thus, human MSC from both young and old donors express significant levels of neurotrophic factors but that some yet to be identified molecules are not produced during the differentiation process in old donor MSC. A recent study showed that neuroectodermal differentiation of MSC is limited in old donors (>45 years) and that the levels of neural stem cell markers, nestin and neuroD, were lower in old donors (Hermann et al. 2010). In contrast, chondrocyte differentiation of the MSC was unaffected by age. However, another study by the same group showed that a panel of neurotrophic factors was up-regulated by the neuroectodermal differentiation protocol (Habisch et al. 2010) but they did not study the effect of age on this process.

In both the human and rat (Caddick et al. 2006) cell co-culture systems, the dMSC promote significantly more neurite outgrowth than that elicited by the MSC. We propose that this increase in neurite outgrowth is the result of the secretion of neurotrophic factors, such as NGF and BDNF, by the human MSC as previously demonstrated for differentiated MSC of rat origin (Mahay et al. 2008b).

Effects of donor age on cultured MSC (Papers I & II)

Many studies have speculated that the number of MSC within the bone marrow and their differentiation capacity declines with age (Sethe et al. 2006). It has been noted that the adult stem cells do suffer the effects of aging *in vivo* resulting in decreased ability to self-renew and properly differentiate (Roobrouck et al. 2008). Aging is thought to be determined by chromosome telomere shortening following cell division; it is well known that cells can escape this process *in vitro* by acquiring mutations in specific genes. It has been reported that telomere length is significantly greater in MSC from young donors than in their older counterparts (Baxter et al. 2004). In addition, telomere length decreases with increase in passage number *in vitro*. Interestingly, there were no qualitative differences in glial marker expression profiles with donor ages of 59, 58 and 32 years old (P1, P2 and P3, respectively). However, a more detailed, quantitative assessment of glial marker expression and molecular characterisation on a larger group of donors of different age and gender is

needed before these cells can be regarded as identical. *In vitro*, MSC have been shown to stop proliferating at passage 40 (Bruder et al. 1997). This is accompanied by an increase in cell size, which is often associated with senescence.

However, MSC from old donors showed reduced levels of the proliferation marker ki67 suggestive of a reduced growth rate (Hermann et al. 2010). In our second study (Paper II) study we calculated the population doubling times by passaging, counting and re-plating a defined number of cells every week up to 9 weeks. We found that cells from young donors maintained their proliferation rates over multiple passages whereas the old donor cells grew progressively slower from week 5. Other studies have shown that MSC from old donors exhibit a decreased maximal life span and accelerated signs of senescence when compared with young donor cells (Stenderup et al. 2003). In contrast, in another study there was no clear association between the maximal number of population doublings and donor age (Wagner et al. 2009). We observed that the old donor MSC became more granular and adopted a fried egg like and flatter larger morphology after time in culture, starting at passage 5-6; these morphological changes are also associated with replicative senescence (Wagner et al. 2008). In order to obtain a suitable number of cells for clinical transplantation it is likely that MSC would have to be expanded for multiple passages and then differentiated to the required phenotype. Previous studies have shown that adipocyte and osteogenic differentiation potential is decreased with passage number (Bonab et al. 2006;Kim et al. 2009) whereas neuronal differentiation does not appear to be significantly affected (Khoo et al. 2008). We found that glial growth factor differentiation of MSC at late passage produced a similar response to cells treated at T3, i.e. further enhanced neurite outgrowth with young but not old donor MSC.

Human MSC and regeneration after peripheral nerve injury (Paper III)

In Paper III we evaluated a novel two component fibrin glue conduit for repair of sciatic nerve injury with 10mm gap. To increase the conduits effectiveness, mesenchymal stem cells of human origin (MSC) were transplanted to support axonal regeneration. In the settings of xenogenic cell transplantation, cyclosporine was added to potentially improve survival of transplanted cells.

Human mesenchymal stem cells survived 3 weeks post transplantation as indicated qualitatively by PKH26 labeling, both in groups treated and untreated with cyclosporine A. It has been suggested that human mesenchymal stem cells might possess immunomodulatory properties and might be uniquely immune tolerated, similarly to rodent and porcine MSCs (Atoui et al. 2008). MSCs are considered MHC class I positive and MHC class II negative, which protects them from NK cell-mediated deletion and allows them to escape recognition by CD4+ T-cells. Moreover, the lack of expression of Fas-ligand and co-stimulatory molecules like B7-1, B7-2 or CD-40 is also advantageous (Pittenger et al. 1999). However, despite evidence that MSC remain hypoimmunogenic in a xenogenic setting as demonstrated by the mixed lymphocyte reaction (Grinnemo et al. 2004), there are conflicting reports on the reaction to MSC transplantation *in vivo* (Grinnemo et al. 2004;Grinnemo et al. 2006), (Atoui et al. 2008) with some authors demonstrating survival of human MSC for 8 weeks and lack of immune rejection in an infarcted myocardium model in immunologically competent animals while according to

others, MSC survive only in immunodeficient animals receiving additional immunosuppression.

In this study, suspended in the fibrin glue matrix, human MSC stimulated axonal growth and increased the distance of regeneration, resulting in presence of the pioneering axons in the distal stump at 3 weeks postoperatively. The positive effect was present only in groups where experimental animals received cyclosporine A in addition to cell transplantation. Similarly, encouraging results have been seen in experiments with application of bone marrow-derived or umbilical cord-derived mesenchymal stem cells differentiated into glial lineage suspended in Matrigel (Matsuse et al. 2010; Shimizu et al. 2007), in combination with the FK506 immunosuppressive agent. However the latter studies lack control group without immunosuppression. On the contrary, in a study where MSC have been transplanted in immunologically competent rats without immunosuppression and favourable results of treatment have been found, the gap between nerve ends was much shorter than in our experiment and no morphological analysis was performed making the studies difficult to compare (Pan et al. 2006).

Cyclosporine A is an immunosuppressive agent with known neuroprotective potential (Ibarra et al. 2007). By inhibiting romatase and calcineurin activity (Sosa et al. 2005) cyclosporine A promotes neuroregeneration and neuronal extension via induction of GAP-43 (Strittmatter et al. 1992) although this effect is less pronounced than for FK506 (Gillon et al. 2003). It is postulated that interaction with calcineurin is not the only mechanism of action of cyclosporine A its analogue, which is not immunosuppressive, also induces DRG neurite extension (Steiner et al. 1997). The dose of cyclosporine in our study (15 mg/kg body weight) was significantly higher than the neuroprotective dose of initially 2.5mg/kg ip followed by 5mg/kg orally (Ibarra et al. 2007). Comparison between groups with and without cyclosporine injections in our study revealed that cyclosporine may have its own effects on axonal regeneration. Addition of cyclosporine increased axonal density 3 mm from the proximal nerve end in the conduit, regardless if cells were transplanted, as indicated by significantly increased area covered by regenerating axons. Other authors have noted similar effects of systemic administration of cyclosporine on extensive sprouting from retinal ganglion cells into peripheral nerve graft (Gillon et al. 2003), extensive arborising and ingrowth from dorsal root into the spinal cord (Sugawara et al. 1999) and non functional axonal growth at the edges of transected spinal cord (Ibarra et al. 2007). Cyclosporin A did not affect the immunological reaction significantly compared to the empty conduit. When adding MSC the macrophage reaction increased significantly but interestingly the effect of immunosuppression in combination with cells showed a dramatic decrease in the immune response.

Rat MSC and regeneration after spinal cord injury (Paper IV)

In Paper IV we demonstrated that differentiated MSC express glial markers and increase production of BDNF. However, the ability of MSC to differentiate along a neural or glial lineage generated controversy since a number of reports have demonstrated very little or no differentiation following transplantation of native

MSC in the central nervous system (Neuhuber et al. 2004;Lu et al. 2004;Castro et al. 2002;Swanger et al. 2005;Yoshihara et al. 2006).

Nevertheless, it has been repeatedly demonstrated that both native and differentiated MSC can prevent secondary degeneration, reduce cavity formation and stimulate axonal regeneration and re-myelination in different spinal cord injury models (Abrams et al. 2009;Kamada et al. 2010;Someya et al. 2008;Osaka et al. 2010;Park et al. 2010;Hejcl et al. 2010;Sasaki et al. 2009;Zurita et al. 2008). Moreover, MSC have been found to enhance angiogenesis in the ischemic boundary zone, stimulate neurogenesis, reduce the infarct size, restore the cerebral blood flow and blood brain barrier, and support neurological functional recovery after traumatic brain injury and stroke in adult rats (Brass 2006;English et al. 2006;Li and Chopp 2009;Chopp et al. 2009).

Neurotrophic factors promote regeneration: The mechanisms of neuroprotective and growth-promoting effects of MSC are largely unknown, but it has been proposed that neurologic benefits resulting from MSC transplantation may come from the increased production of neurotrophic factors in the trauma zone and reduced secondary degeneration and cavitation (Li et al. 2002). The latter hypothesis seems to be in line with the discussion that functional improvements appear relatively soon after injury and MSC transplantation, and can indicate that neuroprotection has been achieved through secretion of trophic factors rather than anatomical restoration due to axonal growth and synaptogenesis (Nandoe et al. 2006).

In recent years, numerous studies have reported that native MSC could produce various growth factors including neurotrophins BDNF and NT-3 (Garcia et al. 2004;Neuhuber et al. 2005;Schuhmann et al. 2005;Crigler et al. 2006;Yaghoobi and Mowla 2006;Chen et al. 2007;Hokari et al. 2008;Karussis et al. 2008). Different expression profiles for chemokines and cytokines in MSC from different donors have been recently reported (Zhukareva et al. 2010). In addition, it has been shown that transplanted MSC can enhance production of endogenous VEGF and GDNF in the reactive host astrocytes (Shen et al. 2010).

It has been repeatedly demonstrated that neurotrophic factors BDNF, NT-3, GDNF and CNTF can rescue axotomized descending and ascending tract neurons from retrograde cell death, support axonal regeneration and reduce secondary degeneration and cavitation in different models of spinal cord injury (Novikova et al. 1996;Namiki et al. 2000;Kobayashi et al. 1997;Houle and Ye 1999;Ruitenberget al. 2003;Tobias et al. 2003;Liu et al. 2002;Deumens et al. 2005). We have previously reported that continuous intrathecal infusion of exogenous BDNF can protect more than 90% of axotomized rubrospinal neurons (Novikova et al. 2002;Novikova et al. 2000). Recently, it has been shown that human MSC can attenuate retrograde degeneration of corticospinal neurons after spinal cord injury (Sasaki et al. 2009) and the present study also showed moderate neuroprotective effect of transplanted dMSC on rubrospinal neurons.

We did not find MSC expressing NT-3 transcripts in our cultures, the present results demonstrated that treatment of MSC with a specific differentiation protocol (Dezawa et al. 2001) significantly increased production of BDNF *in vitro*. However, following transplantation into the injured spinal cord, MSC did not increase expression of neurotrophins BDNF and NT-3 in the host tissue but elevated levels of VEGF. VEGF can provide significant neuroprotection after experimental spinal cord injury (Widenfalk et al. 2003) and a similar effect on VEGF expression has been described after MSC transplantation into experimental stroke model in rats (Chen et al. 2003).

MSC have limited effects on axonal regeneration: Despite the findings that MSC transplants can promote partial functional recovery after spinal cord injury, only limited axonal regeneration across the trauma zone has been reported (Hofstetter et al. 2002;Wu et al. 2003;Ankeny et al. 2004;Himes et al. 2006;Neuhuber et al. 2005;Zurita et al. 2008;Ide et al. 2010). In the present study, dMSC induced axonal ingrowth into the trauma zone but only in some experimental animals regenerated axons crossed the lesion site and entered into the distal spinal cord. It has been demonstrated that MSC can stimulate neurite outgrowth over inhibitory extracellular matrix molecules such as CSPG, MAG and Nogo-A (Wright et al. 2007). Moreover, a combined approach of stimulating the neuronal cell body with cAMP and the injured axon with neurotrophins and MSC transplantation could promote axonal growth into and beyond sites of spinal cord injury (Lu and Tuszynski 2008). We demonstrated that dMSC increased regeneration of rubrospinal axons in the white matter rostral to the injury site but did not increase sprouting of rubrospinal arborizations in lamina V. Sprouting of raphespinal axons in the ventral horn was also reduced. The latter effects support the hypothesis that successful long-distance regeneration after spinal cord injury is accompanied by inhibition of axonal branching (Raisman 2004).

MSC induce sprouting in dorsal horn: In contrast to descending pathways, MSC transplantation caused aberrant sprouting of CGRP-positive sensory axons in lamina III. A similar effect has been shown following transplantation of neural stem cells (Hofstetter et al. 2005). The same authors also demonstrate that suppression of astrocytic differentiation of transplanted neural stem cells prevents graft-induced sprouting of CGRP-positive fibers and allodynia. In our study we found that transplanted MSC significantly attenuated astroglial and microglial activity. The results are in line with previous report that MSC can reduce chronic inflammation and injury-induced sensitivity to mechanical stimuli in experimental spinal cord injury.

Clinical implications

Today the gold standard of nerve grafting is to take an autologous nerve to fill the defect in the trauma zone. Tissue engineering is a promising field to overcome the morbidity that is caused by this process. This study shows that MSC possibly will be of clinical importance in treatment of nerve injuries and the benefits of differentiating the cells into Schwann cell (SC) phenotype to potentially enhance their functionality in clinical settings. This study provides evidence that they have functional characteristics similar to those of SC and most importantly can promote neurite outgrowth.

MSC properties are defined as easy to handle, they have immunomodulation and homing effects, intrinsic self renewal capacity, pluripotency, and multilineage differentiating capacities which makes them a powerful tool in regenerative medicine. In order to obtain a suitable number of cells for clinical transplantation it is likely that MSC would have to be expanded for multiple passages and then differentiated to the required phenotype. Since the techniques vary between different laboratories it is of importance to find protocols that are reproducible in order to decrease the risk of tumor formation. Isolation methods, cell culture plastic, seeding density and medium composition together with additional treatment with growth factors might differ and may influence the properties of the cell functions. Additionally the donors' age and health status might influence the yield, proliferation rate and differentiation potential and therefore must also be taken into consideration (Sotiropoulou et al. 2006a; Sotiropoulou et al. 2006b).

Cells should be harvested for clinical treatment before reaching senescence in order to reduce the likelihood of malignant transformation. The advantage of MSC is their mature state and if they are kept in culture with a low plating density and harvested before they become senescent the risk is minimal. In 2010 there were 100 clinical trials registered to use stem cell therapy with MSC or related cells and some of them are at phase II and III with no adverse effects been reported (Prockop et al. 2010). Cellular treatment in clinical settings is often performed by transplantation directly into the local tissue. The most important aspect in cell therapy should therefore be based on the survival, differentiation and proliferation. Homing by systematic injections or tissue derived cells should further be investigated for helping to expand the clinical treatment alternatives with MSC (Liu et al. 2009).

Rejection of transplanted tissues is a problem and must be overcome with immunosuppressant. MSC can modulate the activation of regulatory T-cells which are thought to have a critical role in the suppression of the immune response. The function is not completely understood but clinical trials with MSC on patients with graft versus host disease (GvHD) has shown promising results (Le et al. 2008); (Ringden et al. 2006). Today MSC are considered as drugs and are restricted under strict regulatory manner and must be manufactured under authorized GMP conditions.

In summary this theses defines new possibilities for treatment of neural injuries with mesenchymal stem cells and tissue engineered constructs, allowing the surgeons to

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bridge nervous defects and promote regeneration following peripheral or spinal cord injuries.

CONCLUSIONS

This present study characterizes the human and rat MSC and investigates the effects of MSC transplantation on regeneration after peripheral nerve and spinal cord injury in adult rats.

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

- Human and rat MSC express characteristic stem cell surface markers, mRNA transcripts for different neurotrophic factors and demonstrate multi-lineage differentiation potential.
- Following treatment with a cocktail of growth factors, both human and rat MSC express phenotypical Schwann cells markers at both the transcriptional and translational level and significantly increase production of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF).
- Undifferentiated human MSC from both young and old donors increase total neurite length of cultured dorsal root ganglion neurons (DRG). Differentiated MSC from the young donors further enhance neurite outgrowth.
- Human MSC from young donors maintain their proliferation rate over 11 weeks and their ability to enhance neurite outgrowth from DRG neurons.
- Fibrin glue conduit with human MSC cells and with immunosuppressive treatment significantly increases the rate of axonal regeneration across the injury gap after sciatic nerve injury and repair in adult rats.
- Transplantation of rat MSC into the injured cervical spinal cord induces up-regulation of VEGF expression in spinal cord tissue and provides neuroprotection for axotomized rubrospinal neurons.
- Cell transplantation attenuates astroglial and microglial reactions, increases axonal ingrowth into the trauma zone, but induces aberrant sprouting of CGRP-positive sensory axons in the dorsal horn of the spinal cord.

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