Schwann cells and mesenchymal stem cells as promoter of

peripheral nerve regeneration

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To my family
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ACKNOWLEDGEMENTS

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ABSTRACT

The transplantation of primary Schwann cells (SC) has been shown to improve nerve regeneration. However, to monitor the survival of transplanted cells within the host, a stable labelling method is required. The in vitro characteristics of green fluorescent protein labelled SC (GFP-SC) and their effects in an in vivo peripheral nerve injury model were investigated. The GFP-SC were readily visualised ex vivo and stimulated significantly better axonal regeneration compared to controls. Clinical use of autologous SC for the treatment of nerve injuries is of limited use due to difficulty in obtaining clinically useful numbers. However, bone marrow mesenchymal stem cells (MSC) can trans-differentiate into SC like cells (dMSC).

The in vitro and in vivo differentiation of MSC was explored, and the study extended to include the easily-accessible adipose stem cells (ASC). In vitro, glial growth factor stimulated MSC to express S100, a SC marker, an expression that maintained following in vivo transplantation. Similarly, untreated MSC transplanted in vivo also expressed S100, which indicates glial differentiation in response to local cytokines and growth factors. Using an in vitro model, comprising dMSC or dASC co-cultured with adult dorsal root ganglia (DRG) neurons, the capacity of the dMSC and SC like differentiated ASC (dASC) to promote axon myelination was verified: both cell types expressed transcripts for protein zero, peripheral myelin protein-22 and myelin basic protein. The potential of stem cells in nerve repair may be limited by innate cellular senescence or donor age affecting cell functionality thus it was essential to determine the effects of donor age on morphology and functionality of stem cells. The proliferation rates, expression of senescence markers (p38 and p53) and the stimulation of neurite outgrowth from DRG neurons by stem cells isolated from neonatal, young or old rats were very similar. However, the distribution and ultrastructure of mitochondria in dMSC and dASC from young and old rats were quite different, and seem to indicate physiological senescence of aged cells. Given the wide-ranging influence of Notch signalling in cell differentiation, including the neural crest to a glial cell type switch, and self-renewal in mammals, its role in the differentiation of stem cells to SC was investigated. The mRNA for notch-1 and -2 receptors were expressed in the dASC, blockage of notch signaling did not affect the neurotrophic and myelination potential of dASC.

In conclusion, these findings show that GFP labelling has no deleterious effect on SC survival and function. MSC and ASC differentiated into glial-type cells acquire SC morphology, and express characteristic SC markers, and the differentiation process was independent of the Notch signaling pathway. Also, following transplantation into a nerve gap injury dMSC improve regeneration. This study established that following co-culture with DRG neurons, dMSC and dASC were able to express peripheral myelin proteins. Also, the functional bioactivity of these cells is independent of the donor animal age. Finally, although the glial lineage differentiated aged cells characterized in this study expressed markers typical of senescence they retained the potential to support axon regeneration.
ORIGINAL PAPERS

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

I. Tohill MP, Mann DJ, Mantovani CM, Wiberg M, Terenghi G.

II. Tohill M, Mantovani C, Wiberg M, Terenghi G.
   Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration. Neurosci Lett. 2004 May 27; 362(3):200-3.

III. Mantovani C, Mahay D, Shawcross S, Terenghi G, Wiberg M
   Expression of myelin proteins by differentiated mesenchymal stem cells

IV. Kingham PJ, Mantovani C, Terenghi G.

V. Mantovani C, Raimondo S, Haneef MS, Geuna S, Terenghi G, Shawcross SG, Wiberg M
   Morphological, molecular and functional differences of adult bone marrow- and adipose-derived stem cells isolated from rats of different ages. (Submitted to Regenerative Medicine)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>α-modified Eagle medium</td>
</tr>
<tr>
<td>Ara C</td>
<td>Cytosine-β-D-arabinoside</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose Stem cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BS</td>
<td>Bottenstein-Sato’s medium</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAMS</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMT-1A/1B</td>
<td>Charcot-Marie-Tooth 1A/1B</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein</td>
</tr>
<tr>
<td>DS</td>
<td>Dejerne-Sottas</td>
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<tr>
<td>ECMM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>bFGF</td>
<td>Fibroblast growth factor beta</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GGF</td>
<td>Glial growth factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>HNK-1</td>
<td>Human natural killer epitope</td>
</tr>
<tr>
<td>LVM</td>
<td>Low viscosity mannuronic acid</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine Double minute 2 protein</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney Murine Leukaemia Virus</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MtT Red</td>
<td>Mitotracker Red</td>
</tr>
<tr>
<td>MVM</td>
<td>Medium viscosity mannuronic acid</td>
</tr>
<tr>
<td>NT-3/4/5</td>
<td>Neurotrophin3/4/5</td>
</tr>
<tr>
<td>ntSC</td>
<td>Non-transduced Schwann cell</td>
</tr>
<tr>
<td>NCAD</td>
<td>N-cadherin</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>Notch</td>
<td>Neurogenic locus notch homolog protein 2</td>
</tr>
<tr>
<td>p38</td>
<td>Protein 38</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGP</td>
<td>Protein gene product</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
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<tr>
<td>PMP22</td>
<td>Peripheral myelin protein 22</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>P0</td>
<td>Protein zero</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SASP</td>
<td>Senescence Associated Secretory Phenotype</td>
</tr>
<tr>
<td>SC</td>
<td>Schwann cell</td>
</tr>
<tr>
<td>SEM</td>
<td>Scansion Electron Microscopy</td>
</tr>
<tr>
<td>SLI</td>
<td>Schmidt-Lanterman Incisures</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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1.0 Introduction

Peripheral nerve injuries are most commonly seen following accidental lacerations, trauma, falls or industrial accidents. Despite the development in surgical techniques functional recovery from such injuries remains limited. The peripheral nervous system is a highly specialised and organised structure which is responsible for relaying information to and from the central nervous system. The fundamental unit is the peripheral nerve fibre which is composed of a cytoplasmic extension of a sensory, motor or sympathetic neuron (the axon) supported by Schwann cells and extracellular matrix components which compose the basal lamina and an inner endoneurial sheath.

Schwann cells (SC), the glial cells of the peripheral nervous system, surround and support axons. In unmyelinated axons, SC support and separate multiple small diameter axons by enveloping them with extensions of the cell cytoplasm. In a myelinated fibre SC produce a multilayered sheath of myelin which wraps an individual axon. The SC nucleus and cytoplasm lies outside the myelin sheath and rests externally on a basement membrane. At regular points the SC cytoplasm comes into direct contact with the axon, slightly constricting the axon at these points to form the nodes of Ranvier. Nodes of Ranvier provide entry points for extracellular ions which facilitate the saltatory propagation of impulses from node to node.

When a traumatic peripheral nerve injury occurs, based on the severity of the injury, the SC start a process called Wallerian degeneration, a complex cascade of events, which involve changes in the neuronal cell body, the axon and its associated SC which ultimately will lead to regeneration (Hall and van Way 1994). Following the breakdown of axonal and myelin constituents at the site of injury SC de-differentiation and proliferation occurs. Cellular debris is removed initially by local proliferating SC in association with the infiltration of macrophages (Hall 1993).

Proliferating SC align to form bands of Bünger between the proximal and distal stump, in addition to this they upregulate the synthesis of a number of important neurotrophic factors. A strict correlation exists between regenerating axons and SC. The latter provide structural and trophic support to regenerating axons, whilst axon-SC contact promotes proliferation, differentiation and consequently myelin formation (Maurel and Salzer 2000). Following injury, SC in the distal nerve stump divide and upregulate the gene expression of the low affinity neurotrophin receptor p75, the neuregulin receptors, NCAM and L1 (Martini and Schachner 1988), laminin, cytokines (Kurek, Austin et al. 1996) and neurotrophic factors.
Simultaneously these changes produce a population of axon-responsive SC, which facilitates axonal growth into the zone of injury. In SC adjacent to regenerating axons a second wave of SC proliferation occurs, this precedes the formation of myelin (Pellegrino and Spencer 1985) and a 1:1 relationship between myelinating SC and axons is established (Jessen and Mirsky 1999).

1.1 Cell adhesion molecules and the extracellular matrix

Cell adhesion molecules (CAMS) are important constituents of the receptor systems present on neuronal growth cones. During growth, axon-SC interactions are mediated by cell adhesion molecules such as neural cell adhesion molecule (NCAM), L1, and N-cadherin, whereas axon-basal lamina contact is mediated by laminin and integrin interactions (Bixby and Jhabvala 1990; Court, Wrabetz et al. 2006). With maturation, this expression is significantly reduced in myelinated fibres, but it is maintained in unmyelinated fibres (Martini 1994; Saito, Nakao et al. 2005).

Signalling from the extracellular matrix, and in particular the basal lamina, plays an important role in supporting SC and axons during development and axonal regeneration (Jessen and Mirsky 1999). The basal lamina contains active molecules, such as laminin and fibronectin, surrounded with collagen matrix, included laminin being the most potent adhesion molecule for promoting axonal outgrowth (Ide 1996; Armstrong, Wiberg et al. 2007). Signalling exchange between axons and basal lamina are mediated by laminin-integrin binding (Letourneau, Condic et al. 1994) which promotes adhesion and motility of growth cones through intracellular signal transductions (Bixby and Jhabvala 1990).

1.2 Nerve repair

The concept behind the development of bio-engineered systems is to simulate as closely as possible the internal structural and molecular characteristics of the tissue being substituted. In vivo studies have shown that the addition of laminin to nerve conduits improves regeneration (Bailey, Eichler et al. 1993; Court, Wrabetz et al. 2006; Armstrong, Wiberg et al. 2007). During the last few decades attention has been focused to the concept of bio-engineered nerve grafts made of a bio-compatible/resorbable material (Schmidt and Leach 2003).

The development of nerve conduits to bridge injury gaps has provided great improvements into the physiological processes that occur in nerve regeneration (Hall 2001). The conduit confines and directs axonal regeneration and prevents the influx of inflammatory cells and scarring. Studies of biological materials started with Glück (1880) who initially applied artery
and vein grafts for nerve conduits. However, the artery and veins are not ideal because there is a limited supply of dispensable autologous vascular material and lack of degradation, and some non-biological materials have also limited clinical applicability due to their lack of degradation.

Poly-3-hydroxybutyrate (PHB) is a biopolymer produced from 3-hydroxypropionic acid and is found as a natural storage product in many bacteria. Following chemical transformation, PHB is polymerised into fine fibres which can be formed into sheets which in turn can be rolled into conduits. PHB is non-antigenic and bio-compatible, being resorbed through hydrolytic degradation over a period of approximately 24 months (Hazari, Wiberg et al. 1999). A number of experimental studies have demonstrated the ability of PHB conduits to support nerve regeneration in vivo (Mosahebi, Simon et al. 2001; Young, Wiberg et al. 2002; Mohanna, Terenghi et al. 2005). A recent clinical trial of PHB conduits for short-gap nerve repair has also shown sensory functional recovery equal, if not better, to epineural suture repair (Aberg, Ljungberg et al. 2009).

1.2.1 Transplantation of Schwann cells
The pivotal role of SC in nerve regeneration is well described. In experimental studies of regeneration through injury gaps, SC transplantation has been shown to improve regeneration due to their ability to release neurotrophic factors, contact guidance through the expression of cell adhesion molecules, and the synthesis of extracellular matrix. The interaction of transplanted SC with regenerating axons and their distribution throughout the conduit has been described following the use of labelling techniques. Labelling of cells transplanted in a bio-engineered system allows the assessment of their survival and integration within the host upon histological analysis. A more stable labelling has been obtained by transfection of cultured SC with the lacZ reporter gene (Mosahebi, Woodward et al. 2000). However, a number of problems have been reported about histological analysis as lacZ expression is confined to the nucleus. For this reason, during the past decade, bioluminescent protein markers have received great interest for labelling and tracking cells, proteins and for measuring gene expression. The first described and most widely studied is the green fluorescent protein (GFP) extracted from jellyfish in the Bay of Naples (Shimomura, Masugi et al. 1978). Green fluorescent protein was discovered by Shimomura et al. (Shimomura, Johnson et al. 1962) as a companion protein to aequorin, a well known chemiluminescent protein that produces blue light in the Aequorea jellyfish.
GFP is a protein of 238 amino acid residues which fold to form an 11 β-stranded ‘barrel’ surrounding a p-hydroxybenzylideneimidazolidinone chromophore. GFPs are stable proteins meaning their spectral properties are unaffected by denaturing conditions. The ‘barrel’ of β-strands surrounding the central chromophore protects this light-emitting region of the protein. The advantage of using GFP to label transplanted cells is that the cells can be visualized in the presence of other fluorochrome-labelled antigens (Zimmer 2002).

1.3 Stem cells applications for peripheral nerve injury
The use of autologous SC for the treatment of acute injuries may be impractical due to the technical difficulties and time required in harvesting and expanding such cells, which may require up to 10 weeks. The ideal ‘transplantable cell’ should be easily accessible, capable of rapid expansion in culture, immunologically inert, capable of long-term survival and integration in the host tissue. Adult stem cells form a suitable alternative to SC. Stem cells are the fundamental cellular building “bricks” of life, in addition to coordinating organ growth from embryo to adulthood they also play an important role in tissue regeneration and repair (Caplan 2007).

During the past decade the field of stem cell biology has developed a remarkable evolution based on reports demonstrating that adults stem cell possess great abilities in tissue regeneration. Many efforts have been focused on understanding the molecular mechanisms that regulate their plasticity and developing ways to exploit this capacity for a therapeutic use. A simplistic definition of the stem cell describes it as a clonogenic cell capable of self-renewal and multi-lineage differentiation (Till and Mc 1961). The characterization of stem cells can cause confusion as there is no universally accepted definition of what a stem cell is and no unified theory describing their origin, plasticity and function in the adult organism (Tohill and Terenghi 2004). These characteristics are that the cell must be (i) undifferentiated (i.e. lacking a tissue-specific differentiation marker), (ii) capable of proliferation, (iii) self-renewable, (iv) able to produce a large number of differentiated functional progeny and (v) able to regenerate tissue after injury (Loeffler, Bratke et al. 1997).

The control of self-renewal and differentiation is most probably influenced by extrinsic factors, i.e. the environment or niche, and intrinsic cellular factors. The environment influences the biochemical and morphological properties of stem cells. Extrinsic factors include cell-cell interactions and the extracellular matrix, on the other hand, intrinsic factors determining self-renewal and division exist (Ferguson and Slack 2003; Caplan and Dennis 2006).
1.3.1 Bone marrow stem cells
A large number of studies have suggested that stem cells from one tissue can cross lineage and differentiate into cells of other lineages either *in vitro* or *in vivo* after transplantation. This plasticity, or ability for cells to trans-differentiate, has aroused great interest for its therapeutic potential in tissue engineering. A promising candidate to display such plasticity is the bone marrow stem cell. Bone marrow contains two distinct populations of progenitor cells: haematopoetic progenitors and bone marrow stem cells progenitors. Marrow stem has been identified as the site of origin for mesenchymal progenitors for bone, cartilage, tendon, adipose tissue and muscle. Marrow stem cells are easily accessible through the aspiration from the bone marrow cavity. They readily adhere to plastic in tissue culture in comparison to their non-adherent haematopoietic counterparts (Bianco, Riminucci et al. 2001).

There is evidence that marrow stem cells are capable of neuronal antigen expression *in vitro* (Dezawa, Takahashi et al. 2001; Kim, Seo et al. 2002) and *in vivo* (Kopen, Prockop et al. 1999; Mezey, Chandross et al. 2000). They have been shown to differentiate into astrocytes following direct transplantation into the rodent brain (Azizi, Stokes et al. 1998). Recent studies described remyelination of spinal cord lesions following intravenous delivery of marrow stem cells and showed that local delivery of MSC at the site of spinal cord injury was associated with the formation of neurofilament bundles at the interface between scar tissue and graft (Akiyama, Radtke et al. 2002; Hofstetter, Schwarz et al. 2002). It is not clear what mechanisms govern the *in vivo* differentiation and migration of MSC within zones of injury; however it is likely that the local milieu of growth factors, cytokines and local stem cells have some influence.

1.4 The myelination process
The study of myelination process started more than one hundred and fifty years ago with great controversy. The discovery of myelinated fibres is credited to Cajal (1909) although many researchers before him describe a “tunic” surrounded the peripheral nerve though it was not clear where that tunic originated. Recent progresses in molecular biology have markedly expanded our knowledge of the molecular mechanisms behind the proliferation and differentiation of SC, the myelin-forming cells in the peripheral nerve system (PNS). Myelin sheaths are formed around axons by extending, biochemically modifying and spiralling plasma membranes of these cells and from olygodendrocytes in the central nerve system (CNS). Immature SC can differentiate into either pro-myelinating SC or non-myelinating SC, depending on the presence of extrinsic stimuli (Mirsky, Jessen et al. 2002).
At the pro-myelinating stage, each SC forms a single segment of myelin around the axon to create a 1:1 relationship with axons increasing the expression of integral membranes proteins. Myelin is a substance rich in protein and lipids that forms layers around the nerve fibres and acts as insulation (Simons and Trotter 2007). It is present in both the central and peripheral nervous systems and its function is to facilitate rapid conduction of action potentials and exchange of information between the nervous system and the organs. In the two types of nervous system the myelin is chemically different (Simons and Trotter 2007), but they both perform the same function, that is, to promote efficient transmission of a nerve impulse along the axons. Segments of compact myelin are called internodes because they are separated by specialized structures called “nodes” where ion fluxes across the axonal membrane generate potentials. It is important to distinguish the constituents of compact myelin from those of other myelin-related membranes in the sheaths. Myelin is composed predominantly of water, lipids and proteins; lipids comprise up to 70%-80% of the dry weight of myelin. The major lipids include: cholesterol, cerebrosides, sulphatides, the phosphatidy lethanolamines, phosphatidylcholines, phosphatidylserines, sfinomyelins and phosphatidylinositols (Snipes and Suter 1995). In the PNS the myelin formed by Schwann cells is based on four integral membrane glycoproteins expressed in myelin internodes, which are specific for myelin and myelin forming cells. The major proteins are protein zero (P0), peripheral myelin protein (PMP22), myelin basic protein (MBP) and myelin associated-glycoprotein (MAG) (Snipes and Suter 1995). The mutations of the genes that code for these proteins can be the cause of some hereditary neuropathies involving myelin formation. Protein zero, peripheral protein-22 and myelin basic protein are components of compact peripheral nerve myelin (Snipes, Suter et al. 1992) while MAG has been localized to the innermost portion of the myelin, adjacent to the axon and to noncompact myelin at the Schmidt-Lanterman incisures and paranodal terminal loops (Martini 1994).

1.4.1 Protein zero
Protein zero (P0) is a 28 kDa protein that is confined to the compact portion of the mature myelin. P0 consists of an extra cellular oriented immunoglobulin-like domain, a single transmembrane domain and intracellular cytoplasmic domain. The sulphated glucuronic acid on P0 in the immunoglobulin-like domain carries the human natural killer HNK-1 epitope that has been implicated in cell adhesion (Voshol, van Zuylen et al. 1996). In the nervous system, the HNK-1 epitope is expressed primarily on glycoproteins that have been implicated in adhesion, such as N-CAM, L1, and MAG, suggesting that it could have a functional role in
cell-cell interactions (Martini and Schachner 1988). P0 is believed to stabilize the intraperiod line of compact PNS myelin by homophilic interactions (Quarles 2002). However, other evidence suggests that part of the homophilic binding is due to protein-carbohydrate interactions between P0 molecules, some of which involve the adhesion and related HNK-1 epitope. It has been found that low basal levels of P0 are expressed in Schwann cells and neural crest cells early in embryonic development before myelination, suggesting that P0 could be an early marker of glial lineage and performing other functions that are unrelated to myelination (Jessen and Mirsky 1999).

The massive up-regulation of P0 by Schwann cells during active myelination appears to involve largely unknown signals from the axon, interaction with the basal lamina and elevated intracellular cAMP. Human neuropathies affecting myelin in the P0 gene mutation include disease such as Dejerine-Sottas and Charcot-Marie-Tooth (CMT) type 1B (Zielasek, Martini et al. 1996).

### 1.4.2 Peripheral myelin protein 22

Peripheral myelin protein 22 is a glycoprotein which has a molecular mass of 22 kDa and comprises 2%-5% of PNS myelin protein in rodents and human (Pareek, Suter et al. 1993). The PMP22 proteins belong to the tetra-family trans-membrane proteins. It is formed by four domains with two extracellular loops, while intracellular the molecule has two cytoplasmic tails and intracellular loop and carrying an L2/HNK1 carbohydrate chain (Muller 2000).

Although P0 is nerve tissue specific, PMP22 is present in other tissues, such as lung, gut and heart. This protein has received attention because abnormalities of its gene can cause the demyelination in several neuropathies in humans. It seems that the protein plays a role in the formation and maintenance of myelin (Muller 2000). The PMP22 gene has been proposed as the CMT-1A disease gene (Suter and Snipes 1995). Interestingly, several P0 point mutations have been found in patients affected by CMT-1B. D’Urso et al. (1999) (D’Urso, Ehrhardt et al. 1999) have shown for the first time the evidence that PMP22 forms complexes with another myelin protein at the plasma membrane. They identified the protein as the peripheral myelin P0. The data presented in their study may explain why alterations in either of the PMP22 or P0 proteins, possibly interfering with the formation of complexes, are sufficient to destabilize the myelin structure leading to the same pathological symptoms.
1.4.3 Myelin Basic Protein
The myelin basic protein (MBP) comprises a class of proteins ranging in molecular weight from 12 to 22 kDa, which arise from a single gene via alternative RNA splicing that result in several isoforms of membranes proteins 14kDa, 17kDa, 17. 2kDa, 18. 5kDa and 21. 5kDa (Snipes and Suter 1995). Furthermore, the MBP gene is a part of a more complex gene structure, the products of which may play a role in oligodendrocyte differentiation prior to myelination. It seems that the new cDNAs products derived from this genes are code for proteins that have functions in a number of cell types unrelated to the myelination process. These new proteins have been defined as “golli proteins” (Campagnoni, Pribyl et al. 1993). Landry et al. (1996) (Landry, Ellison et al. 1996) recognized different regions of different cell types were the “golli” proteins are expressed including: spinal cord, developing cortex, olfactory system and the dorsal root ganglia. Their functions are still not well-known, but the studies involving the MBP genes extend beyond the myelin boundaries. The MBPs are essential for myelin compaction (Yin, Kemp et al. 2001) although their function even in the myelin membrane is still largely unknown. It has been demonstrated that in CNS there is a strict correlation between the expression level of MBP and myelin thickness (Shine, Readhead et al. 1992), but it seemed not to be true in PNS. However, some results confirmed that the relationship between the fibre diameter and the Schmidt-Lanterman Incisures (SLI) is based on the amount of MBP (Smith-Slatas and Barbarese 2000). Martini et al. (1997) (Martini and Schachner 1997) observed that P0 and MBP can play interchangeable roles during myelin formation in the dense line. The conclusion of their study implies that when pro-myelinating Schwann cells do not synthesise MBP, the thickness of the myelin is determined by the level of P0 expression (Martini, Mohajeri et al. 1995).

1.4.4 Myelin associated glycoprotein
The myelin associated glycoprotein (MAG) is a 100 kDa transmembrane glycoprotein that is selectively localized in periaxonal Schwann cell and oligodendrocytes membranes of myelin sheaths, suggesting that it functions in glia-axon interactions in both the peripheral nerve system and central nerve system (Quarles 2007). MAG is a minor constituent of myelin, comprising ~1% and 0. 1% of all proteins in the CNS and PNS, respectively (Yin, Crawford et al. 1998). MAG is important for the normal myelinated axon and its maintenance; in particular, it has important positive functions in glia-axon interactions and myelination. It was first detected in rat CNS (Quarles, Everly et al. 1973) and sub-fractions of myelin and myelin-related membranes soon demonstrated that MAG was enriched in membranous vesicles that
were heavier that lipid-rich fragments of multi-lamellar compact myelin containing most of the myelin basic protein (MBP) and proteolipid protein (PLP) in the CNS (Quarles 2007). This was the first indication that MAG is selectively localized in glia membranes of myelin sheaths as distinct from compact myelin and the reason that term “myelin associated” was included in its name. MAG is a glycoprotein of the Ig-superfamily with significant homology to the neural adhesion N-CAM (Lai, Watson et al. 1987; Salzer, Holmes et al. 1987). Two MAG isoforms with apparent molecular weights of 67 kDa (termed as S-MAG) and 72 kDa (termed as L-MAG) after deglycosylation result from alternative splicing of the primary transcript (Tropak and Roder 1997). As Quarles (2007) described recently, in the CNS the large isoform (L-MAG) is the predominant form in early myelogenesis, whereas the small isoform (S-MAG) increases with maturation, so that the two are present in approximately equal amounts in adults (Butt, Ibrahim et al. 1998). In the PNS, S-MAG is the predominant form at all ages, although small amounts of L-MAG are present early in development. Expressions of the two isoform of MAG have been studied most extensively in rodents, but has also been reported in humans (Miescher, Lutzelschwab et al. 1997).

MAG was also designated siglec-4. This term “siglec” defines a sub group of sialic-acid binding proteins of the immunoglobulin (Ig) superfamily with a characteristic extracellular domain (Schachner and Bartsch 2000). Like P0 and PMP22, MAG in many species contains the adhesion related HNK-1 carbohydrate epitope (Quarles 1997). Recently, the large isoform of MAG has been identified as the functionally important isoform in the CNS, whereas the small MAG isoform is sufficient to maintain the integrity of myelinated fibres in the PNS (Schachner and Bartsch 2000). Another function of MAG is the modulation of neurite outgrowth by neurons, dependent upon age and neuron type. Recently, MAG has been shown to regulate neurofilament spacing and axon calibre in vivo (Yin, Crawford et al. 1998), which may correlate with the effects on neurite outgrowth observed in vitro. Presumably, MAG mediates these effects by binding to an as yet unidentified receptor on the neuron (Sadoul, Fahrig et al. 1990). Whether the expression of MAG binding molecules can be modulated by interactions with myelinating cells, Turnley et al. (1999)(Turnley and Bartlett 1999) showed that in vitro. MAG binding by sensory neurons in culture is modulated by NGF and that binding varies with time in culture and level of neurofilament expression. More importantly, they reported that NGF produced by Schwann cell (SC) can upregulate expression of the MAG binding molecule to maximal levels.
This indicates that NGF may be a regulator of MAG-MAG binding molecular interactions and may be important during myelination, as well as remyelination and nerve regrowth following injury. In the study they also demonstrated that NGF upregulated expression of the MAG binding molecule in a dose dependent manner and SC were able to act as a source of NGF to promote maximal binding of MAG to the sensory neurons. It is also well established that MAG-mediated signalling affects the calibre of myelinated axons by increasing the expression of phosphorylated neurofilaments (Yin, Crawford et al. 1998), but essentially nothing is known about why PNS axons actually degenerate in the absence of this signalling.

1.5 Notch signalling

The Notch gene encodes members of a family of four receptors regulating cell to cell interaction. Notch was discovered by Thomas Hunt Morgan in 1917, and was so named because the Drosophila melanogaster mutant showed cells changes in small notches on the wings. The Notch receptor is a hetero-oligomer composed of an extracellular portion, associated with non-covalent interaction, and a calcium-dependent smaller portion, which includes an extracellular, transmembrane helix and an intracellular part (Figure 1). The Notch cell-surface receptor is activated by its ligands, Delta (Dll-1, Dll-3, Dll-4) and Jagged (Jagged -1 and Jagged-2). Both receptors and ligands are single-pass transmembrane proteins which suggest that signalling occurs through the Notch receptor following cell-cell interaction. Notch target genes, which are linked to Notch signalling, have also been isolated and named Hes-1 (Hairy enhance of split-1), Nuclear factor-κB (NF-κB), Cyclin D1 and c-myc (Miele 2006). The Notch receptor is expressed particularly in stem cells with potential to determine their differentiation toward neural or glial cells type.

![Figure 1. The Notch signaling Pathway](https://kato.y. themultiple roles of Notch signaling during left-right patterning. Cell Mol Life Sci. 2011)
Recent studies showed that Notch may inhibit neuronal differentiation of neural crest cells and promote the acquisition of glial identity (Gaiano, Nye et al. 2000; Morrison, Perez et al. 2000). It has been shown that the inhibitory role of Notch seems to apply during oligodendrocytes differentiation, but a recent investigation has also suggested that Notch irreversibly commits neural crest stem cells to a Schwann cell (SC) fate (Morrison, Perez et al. 2000). Schwann cells are derived from the neural crest and develop through a number of intermediate cell phenotypes (Woodhoo and Sommer 2008). SC precursors are generated from neural crest stem cells and first appear in rat nerves during the 14/15 day of the embryogenesis. During maturation they show an increase in the expression of glial marker like GFAP and S100 and to coincide with their final development in myelinating or non-myelinating Schwann cells with the role of support and maintenance of the peripheral nerve system. Notch signalling seems to influence important roles in many types of stem cells as well as the signals that these cells receive from their environment or “niche” (Aguirre, Rubio et al. 2010). The emerging role of Notch signalling influence has also been described in a variety of mammalian stem cells such as haemapoiteic, intestinal and skin stem cells. Wilson and Radtke (2006) suggested that the Notch functions as a stem cell “gate keeper” by influencing the binary cell fate decision or induction of terminal differentiation processes in invertebrates and self-renewing in mammals.

Recent studies have focused on the microenvironment surrounding the stem cells, which sustain different external signals to influence stem cells activities. The idea of a niche as an ideal location for stem cells was proposed initially as a system regulating the hematopoietic precursor cells (Schofield, McDonald et al. 1978). Since then the definition and the characterization of the niche has evolved and presently the description of the niche imply the involvement of the functional regulation of stem cells by both cellular and extracellular matrix constituents of the niche (Jones and Wagers 2008). In the epidermal tissue it has been showed that in the hair follicle Notch influences the ability of differentiating stem cells to a particular cell type (Demehri and Kopan 2009) while in the gut Notch stimulates proliferation and regulates alternative stem cell differentiation (Fre, Huyghe et al. 2005). Furthermore, it has been demonstrated that different stem cells have different life spans often connected to chromosome stability, oxidative stress, telomere length and DNA damage repair activity (Chen, Lin et al. 2006; Rossi, Bryder et al. 2007; Rai, Onder et al. 2009). Most of the adult stem cells have limited self-renewal and proliferative potential which decrease with the advancing age of the organism (Rossi, Jamieson et al. 2008). To avoid the possibility of
extinction of stem cells pool, adult stem cells may be maintained in the quiescence state, which is a reversible cellular state connected to the self-renewal characteristic (Asai, Liu et al. 2011). Interestingly, Notch appears to be involved in the restoration of regenerative potential of aged muscle where in response to an injury the satellite cells, situate in the basal lamina of the muscle fibers, are re-activated from their quiescent state and are stimulated to proliferate. This activation is regulated by Notch signalling pathways, as shown by the correlation between the decreasing of Notch signalling and the lack of regeneration in the damaged muscles; moreover, the decline of the regeneration could also be reverse by improving Notch expression and activation (Conboy, Conboy et al. 2003). Given the wide ranging role of Notch, it was important to this study to investigate the expression of Notch during the differentiation of adult stem cells to Schwann cells. Furthermore, we compared Notch expression in adult stem cell originating from neonatal, young adult and aged rats.

1.6 Aging and senescence markers in stem cells biology

Generally, stem cells remain in a quiescent state until injuries or diseases occur to disrupt the tissue. The stem cells are then induced to re-activate their cell cycle through signal stimulation in order to promote the regeneration or repair processes while other somatic cells remain inactivated. This hypothesis suggests that stem cells are capable of auto-regulation of their cells cycle whereas somatic cells are not (Rubin, Yandell et al. 2000). However, it is still unclear if stem cell activation is influenced by extracellular signals from within the “niche” and if this stimulation is controlled by the aging process of the body. Early studies on cellular senescence showed that somatic cells can divide for a limited number of times when maintained in culture (Hayflick 1965). The maximum number of cell cycles varies according to cell and tissue type: this has been named the “Hayflick number”. When the maximum value of this index is achieved, the cells reduce replication and remain viable, but they are not capable of re-entering the cell cycle regardless of the stimuli they may receive from their surrounding environment. This senescence state is defined as the process during which the cells can no longer divide and it is distinct from the quiescent state, where cells are temporarily non-proliferative but can be induced to divide by the addition of appropriate stimuli. These two distinct processes appear to be very important in the ageing process.

In order to evaluate the relationship between aging and senescent cells different markers have been proposed to identify the terminal cycles of cells in tissue samples from different ages. One of the proposed markers is β-galactosidase staining of lysosomal enzymes, which are
involved in the removal of the galactose residue from glycoproteins, sphingolipids and keratan sulphate in the most cells (Dimri, Lee et al. 1995). In vitro, β-galactosidase is expressed by several types of senescent cells and its expression appears to increase with age in human skin cells (Going, Stuart et al. 2002). However, it is still an open debate whether or not this marker is a useful tool for an early senescence or a long quiescent stage, because it does not seem to be sufficiently specific to distinguish between a reversible and a terminal step in the cell cycle.

Telomerase length is also been suggested as a marker for cellular senescence. Telomeres are defined as the ends part of eukaryotic chromosome and consist of short nucleotide repeats. As it is not possible to replicate the final ends of DNA molecules, the telomeres become shorter after each cell division and their reduced length is indicative of the proliferative stage of the cell. For this reason, the measure of the telomerase activity provides an early marker for cellular senescence (Belair, Yeager et al. 1997). During the last few years researchers have investigated the pathways involving telomeric shortening as well as the upregulation of p53, p38, c-myc and other senescence markers. When p53 is activated, it appears to send signals to the cells that stop their replicative cycles; it acts largely through the induction of the cyclin-dependent kinase inhibitor p21\textsuperscript{waf1/cip1} (Kiyono, Foster et al. 1998).

Adult stem cells derived from bone marrow and adipose tissues have the ability to self-renew and differentiate in different lineages. However, adult stem cells are influenced by the effects of the aging process resulting in the decreasing potential of proliferation and differentiation (Roobrouck, Ulloa-Montoya et al. 2008). The loss of functionality of stem cells isolated from animals of different ages is accompanied by a range of age-related biochemical changes in their niche tissue. Cells from aged animals have shown cumulative oxidative damage and impaired mitochondrial functions. These and other aged-dependent changes in cellular components lead to physiological changes and failure in responses to injury; for example, there is failure in the remyelination process in the central and peripheral nerve systems in demyelination conditions (Franklin 2002). This variation in the functionality of the resident stem cells can be attributed to a decline in the effectiveness of the regenerative response to the environmental cues, and to a lack of response to extrinsic signals that normally lead the stem cells to participate in tissue repair.
1.6.1 Mitogen-activated protein kinase p38

The mitogen-activated protein kinase (MAPK) p38 is strongly activated by stress, but it also appears to play an important role in immune response as well as in the regulation of cells survival and differentiation (De Paula, Bentley et al. 2007). MAPK p38α, also known as RK, CSBP and SAPK2a, was initially described as a 38-kDa protein that mediated the inflammatory response of several cytokines. Three other p38 MAPK family members were subsequently designated p38β, p38γ and p38δ. Recent evidence has lead to the hypothesis that p38 is a tumor suppressor; this is based on the fact that it regulates cell cycle progression as well as mediating apoptosis induction (Bulavin and Fornace 2004). It has been suggested that p38α can negatively regulate cell cycle progression both at the G1/S and the G2/M transition and several mechanism have been implicated (Lavoie, Rivard et al. 1996; Bulavin and Fornace 2004; Yee, Paulson et al. 2004). The involvement of p38α in the G2/M phase cell cycle arrest results from stimuli such as stress, ultraviolet light, methylating agents, osmotic shock and histone deacetylases is documented in many studies (Engel, Schebesta et al. 2005; Mikhailov, Shinohara et al. 2005; Pedraza-Alva, Koulnis et al. 2006). The role of p38 MAPK signalling in cellular responses is diverse and dependent upon the cell type and stimulus. For example, p38 MAPK signalling has been shown to both promote cell death and enhance cell growth and survival (Juretic, Santibanez et al. 2001; Liu, Kato et al. 2001). Wong et al. (2009) studied the role of p38MAPK by generating a dominant-negative allele (p38 (AF)) in which activating phosphorylation sites Thr180 and Tyr182 are mutated.

Heterozygous p38 (AF) mice show a marked attenuation of p38-dependent signalling and age-induced expression of multiple cell cycle inhibitors in various organs, including pancreatic islets. As a result, aged p38 (AF+/) mice show enhanced proliferation and regeneration of islets when compared to aged wild-type. They also investigated an age-related reduction in expression of the p38-specific phosphatase Wip1 (wildtype p53-induced phosphatase-1), which was originally identified as a p53-regulated gene. Wip1-deficient mice show decreased islet proliferation, while Wip1 over-expression rescues the aging-related decline in proliferation and regenerative capacity (Wong, Le Guezenec et al. 2009). The mechanism of p38MAPK activation with advanced age remains unclear; however, Wip1 may serve as a potent physiological regulator of p38 signalling in the process of aging (Wong, Le Guezenec et al. 2009). A role for p38MAPK in experimentally modulated aging has been suggested in several cell types, including hematopoietic stem cells (Ito, Hirao et al. 2006).
Substantial experimental evidence has identified Wip1 as an oncoprotein and a negative regulator of several tumor suppressors, including p38MAPK, p53. However, new findings highlight the benefits that Wip1 confers in maintaining the reservoir of proliferating cells, including adult stem cells and progenitors, thus ameliorating some of the detrimental effects of aging (Chew, Biswas et al. 2009).

1.6.2 The p53 protein: apoptosis leader or quiescent inducer?
Protein p53 is normally expressed at a low level in the cell due to the action of an ubiquitin ligase called MDM2, which promotes p53 degradation. p53 is activated by various stimuli such as stress, DNA damage or inappropriate expression of oncogenes, which lead to tumorigenesis and apoptosis (Batchelor, Loewer et al. 2011). When the binding between p53 and MDM2 is disrupted by these stimuli, the accumulation of p53 in the nucleus modifies the expression of genes that induce either apoptosis or transient (quiescent) or permanent (senescence) cell cycle arrest. It has been demonstrated that p53 removes cells with potentially cancer-promoting activity by inhibiting their growth (Hede, Nazarenko et al. 2011). The mTOR (mammalian target of rampamycin) protein is cytoplasmatic kinase that is generally over expressed in cancer cells (Menon and Manning 2008) and it is required for the activation of cell senescence. Deactivation of mTOR by rampamycin prevented senescence, instead causing cellular quiescence (Demidenko, Zubova et al. 2009). Different studies have shown that there is a cross-talk between p53 and mTOR signalling pathways. Blagosklonny and colleagues (2010) noted that p53 induction does not always lead to cell senescence thus questioning the role of p53 in the senescence process; rather it may indicate that p53 suppresses senescence and promotes cellular quiescence. The suppression of senescence was attributed to p53 mediated inhibition of mTOR activity (Korotchkina, Leontieva et al. 2010). In vivo experiments have shown that p53 is involved in cell differentiation as well as in the cell-fate decisions that occur during development of the nervous system. A few studies have shown that p53 reaches a maximum level of mRNA expression during embryonic development in several tissue including early neuronal precursor cells of the brain in the mouse (Schmid, Lorenz et al. 1991). In addition to stress signals originated from telomere dysfunction, aberrant oncogenic activities are also inducing cellular senescence. Interestingly, prolonged expression of an active Ras protein (Rat Sarcoma protein), H-RasV12, provokes an acute permanent cell cycle arrest, termed 'premature senescence', which is indistinguishable from replicative senescence. While c-myc activates p53 to promote apoptosis, oncogenic Ras induces p53 to initiate premature senescence. It has been shown that oncogenic Ras arrests
The role of mitochondria in cell function

Mitochondria are central components of cells that generate the majority of energy from nutrients. During their normal activity, they generate unstable reactive oxygen species (ROS) which may damage both the mitochondrion and other components of the cell (Navarro and Torrejon 2007). The ROS induced damage plays an important role in aging. Mitochondria are double-membrane organelles, typically rod-shaped, and about 1 micrometer in length. The
number of mitochondria in a cell is determined by the specific function of the cell and its energy needs. Cells such as heart muscle cells have many mitochondria whereas red blood cells have none. The inner mitochondrial membrane is folded into cristae that project inward into the matrix. The region in between the inner and outer membranes is termed the intermembrane space. Each mitochondrion contains 4 to 5 copies of its own circular DNA (mtDNA), which encode ribosomal RNA, tRNAs, and a few of the protein components of the electron transport chain; the other mitochondrial proteins are encoded by the cell nucleus and transported into the mitochondrion via specialized protein import processes.

![Diagram of mitochondria](image)

Figure 2 Schematic of mitochondria

Mitochondria are crucial to the physiology of the cell as they supply energy in the form of ATP, but they may also become involved in the process of cell death (Youle and Karbowski 2005). The population of mitochondria within a cell appear as either a continuous thread-like shape (mitochondrial fusion) or short fragmented forms (mitochondrial fission) (Chan 2006). Mitochondrial fission has been implicated in the cell death process as mitochondrial fragmentation precedes cytochrome C release which initiates the apoptotic cascade reaction (Barsoum, Yuan et al. 2006). However, over-expression of Mitofusin 2 (Mfu2), a mitochondrial fusion-related protein, exhibit protection against ROS and DNA damage-induced cell death (Kuwana, Mackey et al. 2002). Hence, inhibition of mitochondrial fission may promote cell survival (Cheung, McBride et al. 2007). The mechanism involved in the
regulation of mitochondrial fission and fusion is still not completely clear, but these antagonistic events may also be involved in the process of aging and senescence (Navarro and Torrejon 2007). Indeed, mitochondria play an essential role in the life and death of all cells of most tissues and their function is fundamental in the process of cells differentiation (Garrido and Kroemer 2004; Mammucari and Rizzuto 2010; Rehman 2010). Self-renewal of stem cells frequently entails a resistance to the cellular senescence seen in mature differentiated cells, which lose their ability to proliferate after a specific number of cell divisions (Erster, Mihara et al. 2004). Most of the currently identified regulators of stem cell fate are transcription factors and cell cycle regulators, such as Oct-4, Nanog, and c-myc and the associated downstream signalling pathways (Singh and Dalton 2009). Although recent studies suggest that mitochondrial activity may also represent an important regulatory mechanism that helps direct stem cell fate, the role of mitochondria in stem cells has not been well established. In addition to their traditionally described anabolic/catabolic roles such as the production of ATP or synthesizing lipids, mitochondria also appear to regulate a variety of cellular processes, such as cell proliferation and aging in many cell types (McBride, Neuspiel et al. 2006).

The regulatory role of mitochondria is achieved through the controlled release of multiple signalling molecules including ROS and calcium (Gutterman 2005). Unstable ROS are capable of damaging many types of cellular components. It is thought that damage that may accumulate over time from ROS generated from aerobic respiration and may play a significant role in aging (Navarro and Boveris 2007). The ability to repair mitochondria damaged by oxidative stress is crucial in the maintenance of functional mitochondria and in avoidance of entering the cell death cycle (Nisoli, Falcone et al. 2004).

A study conducted in primate adult stromal cells derived from the adipose tissue reported that low passage number cell cultures containing a high proportion of undifferentiated stem cells show significant perinuclear clustering of mitochondria when compared to late passage cells (Lonergan, Brenner et al. 2006). A study conducted on embryonic stem cell undergoing differentiation has shown high level of mitochondrial activity which maybe due to mitochondrial DNA replication (St John, Facucho-Oliveira et al. 2010). The differences seen among the studies of adult stem cells may reflect the properties of the tissues from which the adult stem cells were derived; also, the degree of mitochondrial activity in adult stem cells maybe strongly dependent upon the target lineages into which these cells differentiate.
1.7 Aims of the project

It is well recognized that SC are essential for nerve regeneration and myelin formation. Our previous studies have shown that bone marrow derived mesenchymal stem cells (MSC) and adipose derived stem cells (ASC) can be differentiated along a Schwann cell lineage (Tohill and Terenghi 2004; Caddick, Kingham et al. 2006; Kingham, Kalbermatten et al. 2007) that have functional properties and growth factor synthesis activities similar to those of SC (Caddick, Kingham et al. 2006; Mahay, Terenghi et al. 2008). Previous studies have demonstrated that in co-cultures of embryonic DRG and SC it was possible to reproduce in vitro the formation of myelin (Martini 1998). The signalling pathways involved in the differentiation of ASC are unclear. Notch receptors were investigated as it is known they are involved in the switch of neural crest cells to a glial cell type. Finally, MSC and ASC derived from neonatal, young or old animals were compared to assess possible differences that may affect their use for nerve regeneration.

The aims of this study were to:

- Establish a protocol for labelling SC with green fluorescent protein to aid their identification in combination with histological immunofluorescent staining used to assess peripheral nerve regeneration.

- Assess the ability of marrow stem cell progenitors to undergo glial differentiation in vitro, characterise this differentiation at a molecular, phenotypic and functional level, and to assess the ability of transplanted marrow stem cells to support peripheral nerve regeneration through a PHB conduit in vivo.

- Demonstrate that MSC and ASC differentiated into SC-like cells are able to express the main proteins that are fundamental for myelin formation in the PNS: this property is of crucial importance if MSC or ASC should be used as cell transplants to enhance peripheral nerve regeneration.

- Define the signalling pathway during stem cell differentiation towards a SC-like cell, in particular looking at the role of Notch receptors.

- Determine the morphological, molecular and functional differences of SC, MSC and ASC isolated from rats of different ages.
2.0 Material and Methods

2.1 Schwann cells harvesting

Schwann cells (SC) were harvested from the sciatic nerves of 1-2 day old neonatal Sprague-Dawley rats (Brockes, Fields et al. 1979; Mosahebi, Simon et al. 2001). The animals were killed by cervical dislocation. All procedures were carried out according to the Home Office Act 1986. Under an operating microscope (Zeiss, X10 magnification), a skin incision was made across the dorsum of the animal in the lumbar region, the sciatic nerve was gently dissected out and placed in chilled Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, UK) with 0.1% (v/v) penicillin streptomycin (Invitrogen, UK). Following harvest, the nerves were digested in 2ml of fresh DMEM plus 500µl of collagenase type I (Worthington, USA) and 250µl of trypsin (Invitrogen, UK) at 37°C for 15 min. The medium was aspirated and replaced with 2ml of fresh medium and enzymes; this cycle was repeated twice more. At the end of the last cycle, 10ml of cell growth medium DMEM containing 10% (v/v) foetal bovine serum (Invitrogen, UK) and 0.1% (v/v) penicillin streptomycin was added to neutralize the enzymes and prevent damage to the SC. The cellular debris and undigested fragments of tissue were removed by passing the cell suspension three times through a 21G needle, three times through a 23G needle and finally through a 70µm filter (BD Falcon, UK). The suspension was centrifuged at 600xg for 5 min and the medium aspirated from the pellet. The cell pellet was resuspended in 5ml of fresh growth medium (DMEM; Invitrogen, UK) and the cells plated on poly-D-lysine (Sigma-Aldrich, UK) -coated 75cm² flasks (BD Falcon, UK) and incubated at 37°C, 5% CO₂.

2.2 Schwann cell purification and maintenance

Twenty-four hours after plating the SC were checked for cell attachment to the flask and infection using an inverted microscope (Olympus IX51). The medium was aspirated and the cells washed with DMEM/HEPES (Invitrogen, UK). The medium was replaced with 5ml of fresh cell growth medium containing 10µM cytosine-β-D-arabinoside (Ara C; Sigma-Aldrich, UK), which is cytotoxic and causes the death of proliferating cells (mainly fibroblasts). At this stage the culture is a mixture of SC and fibroblasts, the latter proliferate more rapidly than SC and are therefore more sensitive to the cytotoxic effects of Ara-C. The culture was inspected for fibroblast over-growth and the medium replaced with fresh cell growth medium containing Ara-C; this was repeated for a further 2 days.
The number of days of culture that required Ara-C addition varied depending on the number of fibroblasts seen and SC death. If significant SC death was seen shortly after Ara-C addition then to immuno-depletion of fibroblasts was done immediately.

2.3 Fibroblast immuno-depletion

When the SC-fibroblast culture was sub-confluent the culture was trypsinised by first aspirating the medium and then washing with 5ml of HBSS medium (Invitrogen, UK) to remove traces of serum, which inhibit the trypsin activity. The HBSS medium was removed and 0.25% trypsin/EDTA (2ml for a 25cm² flask or 3ml for a 75cm² flask) was added and the culture incubated at 37°C for 5min. The flask was then lightly tapped to detach the adherent cell and the culture examined under an inverted microscope to check for SC detachment. Once the cells were fully detached, 5ml of cell growth medium was added to neutralise the trypsin. The cell suspension was transferred to a 15ml Falcon-type tube (Starlab Ltd, UK) and centrifuged at 200xg for 5mins. The supernatant was aspirated and the resulting cell pellet resuspended in 500µl of mouse anti-rat Thy 1.1 (1:500 in DMEM; Serotec, UK) and incubated at 37°C for 10min. Thy 1.1 antibodies bind specifically to fibroblasts and, in combination with complement, cause cell lysis. Fresh rabbit complement (Serotec, UK) was prepared in distilled water 8Sigma, UK) 250µl were added to the cell suspension and incubated for 30min at 37°C with gentle agitation every 10min. Cell growth medium (10ml) was then added to the cell suspension and the cells pelleted by centrifugation at 200xg for 5min. The supernatant was aspirated and the cell pellet resuspended in 5ml of SC growth medium (1g/L glucose DMEM; 10% FCS; 1% (v/v) penicillin-streptomycin; all from Invitrogen, UK) and plated into a PDL-coated 25cm² flask (BD BioCoat™, VWR, UK) and placed in an incubator at 37°C, 5% CO₂. Following immuno-depletion, SC purity should be 99% (Brockes et al., 1980). Fibroblast contamination was detected by the morphological differences between the cell types: SC are spindle-shaped bipolar cells which align parallel to each other while fibroblasts have large granular cell bodies, numerous short cytoplasmic processes and distinct nuclei under phase contrast microscopy. If fibroblast overgrowth recurred in culture the immuno-depletion process was repeated. Once SC cultures were confluent they were washed with HBSS medium, trypsinised with 0.25% trypsin/EDTA for 5min at 37°C, resuspended in 10ml of medium and centrifuged at 200xg for 5min. Prior to centrifugation, a sample was taken to determine cell number using a haemocytometer. The cell pellet was resuspended in SC growth medium and plated in a PDL-coated flask (5x10⁵ cells/25cm² flask; 5x10⁶ cells/75cm² flask) and returned to a 37°C, 5%CO₂ incubator: each
flask was labelled with cell type, date of harvesting, date of immunodepletion and passage (trypsinisation) number.

2.4 Bone marrow stem cell harvest
Bone marrow stem cells (MSC) were harvested from 3 adult male inbred Sprague-Dawley rats. Following termination of the animal, the lateral aspect of each hind limb was collected and washed with alcoholic chlorhexidine (Ecolab, UK). The skin was split and the muscle dissected to expose the femur and tibia of each limb. The capsule of the hip joint was opened and the head of the femur was dislocated from the acetabulum. Muscle was stripped from the femur, and the femur removed. Similarly with the tibia, muscles were stripped away from the bone and the distal end of the tibia was dislocated from the talus after division of the ankle ligaments. The bones were placed in chilled α-MEM containing 1% (v/v) penicillin/streptomycin and transported to the tissue culture laboratory. In the class II tissue culture cabinet the distal ends of the long bones were removed using sterilised bone nibblers (Fine Science Tools, Germany) to reveal the marrow cavities. The marrow was harvested from each bone by flushing 5ml of MSC growth medium (α-MEM; 10% FCS; 1% penicillin/streptomycin; Invitrogen, UK) through each marrow cavity using a 5ml syringe and a 21G needle. The aspirated medium and marrow was triturated three times through a 21G needle followed by filtration through a 70μm filter to remove any bone fragments and other debris. The cell suspension was centrifuged in a chilled (4°C) centrifuge at 200xg for 5min. The supernatant was aspirated and the cell pellet was resuspended in 20ml of MSC growth medium and a 10ml aliquot of the suspension plated into a 75cm² tissue culture flask. The cultures were incubated 37°C, 5% CO₂ incubator. After 24h, the flasks were examined under an inverted microscope. The cells present at this stage were a mixture of adherent marrow stem cells, non-adherent haematopoietic cells, dead cells and debris. The non-adherent cells were removed aspirating the growth medium and washing the culture three times with α-MEM. After washing, 10ml of MSC growth medium was added and the cultures returned to the incubator. Washing was repeated every 24h until all non-adherent cells had been removed. The adherents SC were left to reach confluence with medium changes every 72 h. The confluent SC cultures were trypsinised, counted and re-plated at a density of 3.75x10⁵ cells/75cm² flask; cells were also stored in liquid nitrogen in 90% (v/v) foetal calf serum (FCS; Biosera Ltd, UK) supplemented with 10% (v/v) dimethyl sulphoxide (DMSO; Sigma) at each passage stage to create back up stocks.
2.5 Adipose-derived stem cell harvest

Adipose-derived stem cells (ASC) were isolated from the stromal vascular fraction (SVF) of homogenized adipose tissue from Sprague-Dawley rat. The visceral fat encasing the stomach and intestines of adult rats was dissected and chopped to a fine consistency using a sterile razor blade in a fresh, sterile Petri-dish. The tissue was then transferred into a sterile 50 ml tube containing 15 ml freshly made collagenase type I solution and placed in a static water bath at 37°C for 1-2 hours. The enzymatic digestion was then stopped by the addition of an equal volume of stem cell growth medium and the suspension centrifuged at 900 rpm for 5 min. At this stage, an upper layer of floating adipose cells was observed and a pellet of cells constituting the stromal fraction, which contains the stem cells, was formed. The upper layer was carefully aspirated and discarded leaving a pellet of stromal cells. The pellet was resuspended in 10 ml SCGM and the suspension passed through a 70µm filter to remove any large pieces of undissociated tissue. The filtrate was then transferred to a fresh 15 ml tube and centrifuged at 900 rpm for 5 minutes and the resulting pellet was resuspended in 10 ml SCGM and plated into 75 cm² tissue culture flasks. The cultures were maintained at sub-confluent levels in a 37 °C incubator with 5% CO₂, and trypsinised and split as required.

2.5.1 Cell culture harvest for aging study

Adult Schwann cells (SC), mesenchymal stem cells (MSC) and adipose stem cells (ASC) were harvested from Wistar rats of three different ages: new born (neonatal pups), 10 months (young adult) and 20 months (old) using the protocols described in paragraphs 2.1 through 2.5.

2.5.2 Stimulation of glial differentiation of bone marrow and adipose stem cells

MSC and ASC were stimulated towards glial differentiation using the following protocol (Dezawa, Takahashi et al. 2001; Kingham, Kalbermatten et al. 2007). Sub-confluent MSC and ASC were cultured in stem cell growth medium (SCGM) containing 1µM β-mercaptoethanol for 24h. After 24h, the cells were washed and the medium replaced with fresh SCGM supplemented with all-trans-retinoic acid (35ng/ml) for 3 days. The cells were then washed and the medium replaced with SCGM supplemented with platelet-derived growth factor (PDGF; 200ng/ml; Peprotech, UK), basic fibroblast growth factor (bFGF, 10ng/ml; Peprotech, UK), 5µM forskolin (Sigma, UK) and glial growth factor 2 (GGF2; 126ng/ml, Acorda Therapeutics Inc. , USA), and incubated for 14 days with medium changes every 72h.
2.6 Cell transduction with Green Fluorescent Protein

Retroviral transduction is the most efficient means of introducing foreign genetic material into a cell. To transduce cells with green fluorescent protein (GFP) a Moloney Murine Leukaemia retrovirus (MMLV) containing the GFP sequence was used. A mouse embryonic fibroblast cell line (PT67) was used to package the virus and produce a high viral titre. The retroviral genome consists of a single strand of RNA, which is transcribed into double-stranded DNA by the enzyme reverse transcriptase. The double-stranded DNA, known as the provirus, integrates irreversibly into the cellular DNA and is hence replicated along with the host cell genome. PT67 cell lines contain exons for the protein core of the virus (gag), reverse transcriptase (pol), envelope proteins (env) and the packaging sequence (ψ); however the retrovirus produced is deficient in the packaging sequence so that when it infects a target cell it is incapable of replicating (replicative incompetent), thus preventing transduced cells from infecting other cells.

2.6.1 Production of MMLV-GFP-PT67 cell line

The MMLV-GFP-PT67 cell line was produced by D. Mann (Queen Victoria Hospital, East Grinstead, UK) using the following protocol. The ViraPort™ retroviral reporter vector pFB-hrGFP (Stratagene), containing the humanised form of green fluorescent protein from the sea pansy, Renilla reniformis, was used to transfect a RetroPack PT67 packaging cell line (Clontech) for higher titre viral production. Co-transfection of 15µg of pFB-hrGFP and 1.5µg pSV2neo was carried out according to the manufacturer’s instructions using Lipofectamine Plus™ reagent (Gibco, UK). Transfected producer cells were selected for the neomycin pSV2neo resistance marker using 800µg/ml Geneticin (G418 sulphate; Invitrogen) for three days. Cells were continually cultured under selective pressure in DMEM with GLUTAmax™, sodium pyruvate and 4.5 g/ml glucose DMEM (Invitrogen) and 10% (v/v) FCS. Fluorescent colonies were isolated, expanded and screened for the production of virus containing the hrGFP protein. PT67/pFB-hrGFP Clone A2 was reselected using HAT supplements (Invitrogen, UK) according to the Clontech protocol to ensure the retention of the viral genes.

2.6.2 Cell transduction

PT67 cells were grown in 75cm² flasks with medium comprising DMEM high glucose (Invitrogen, UK) 10% (v/v) fetal calf serum and penicillin and 1% (v/v) streptomycin. At 70% confluence the medium was changed to transfer the growth medium to the target cell from the cell type to be transfected growth medium and the cells were transferred to 32°C incubator
and incubated for 72h. The medium (containing a high concentration of retrovirus) was collected and filtered through a 0.45μm filter to remove any suspended PT67 cells. The filtrate was added to a 70% confluent culture of target cells (SC or MSC), and the cells placed in an incubator at 32°C for 24h. The target cells were then placed in their normal growth medium and incubated at 37°C for 24h. This cycle was repeated with the same target cells twice more, making a total of 3 transduction cycles. The target cells were assessed for successful transduction by examining for green fluorescence under blue light using a fluorescence microscope (Olympus BX60). Afterwards, normal, virus-free medium was added to the transduced cells and incubated for 24h at 32°C. This medium was removed, filtered and added to a second batch of non-transduced cells (test cells) and cultured for 24h. This step was performed to assay for live virus particle production by the transduced target cells: any live virus particle shed into the growth medium would transfected the test cells and lead to their expressing GFP.

### 2.6.3 Measurement of transduction efficiency

To measure transduction efficiency (percentage of cells transduced per total number of cells), transduced cells were trypsinised and plated on a chamber slide (Nunc-Labtek, UK) and incubated at 37°C in the appropriate cell type-specific growth medium for 24h. The medium was then replaced with fresh medium containing 10μl/ml of Hoechst (Sigma, UK) which labels cell nuclei and incubated at 37°C for 15 min. The chamber slide was then washed with PBS, covered with fluorescence mounting medium (Vectashield®) and a coverslip, and then examined under a fluorescence microscope (X20 high power field). A digital image was captured of cell nuclei under a rhodamine filter; the filter was changed to acquire a corresponding image of GFP-transduced cells in the same field. The images were transferred to image analysis software (Image-ProPlus®, Media Cybernetics, USA) in which the total number of cells was counted by counting blue Hoechst-labelled cell nuclei. The number of GFP-positive cells was counted and the transduction efficiency calculated: this process was repeated in 10 randomly chosen high power fields.

### 2.7 Cryopreservation of cells

Cells were frozen at regular intervals to build up a stock for further use and analysis. Confluent, cultured cells were trypsinised, diluted in 10ml of cell growth medium and sampled for counting. The cells were divided into aliquots containing approximately 2x10⁶ cells per 1ml aliquot which were then centrifuged at 200xg for 5min: 2x10⁶ is the minimum
number of cells/ml that should be frozen: lower cell concentrations yield fewer surviving cells on thawing. The resulting cell pellets were then suspended in 1ml of chilled cell freezing medium and placed in cryovials for freezing. The active constituent of cell freezing medium is dimethylsulphoxide (DMSO, Sigma, UK) which prevents intracellular ice crystal formation upon freezing. Above 5°C DMSO is toxic to cells and should always be chilled prior to use as a cryopreservative with cells. The cryovials were transferred to an isopentane flask which was placed in a -80°C freezer overnight to allow slow freezing of the cells. The following day, the cryovials were transferred to the gas phase compartment of a liquid nitrogen cryostore for long-term storage.

2.8 Alamar Blue™ assay
Alamar Blue™ (Serotec, UK) was used to measure the metabolic activity of non-transduced and GFP-transduced SC in vitro. Alamar Blue™ is a colorimetric growth indicator solution that changes colour in the presence of REDOX reactions (blue to red) which occur during cell metabolism with colour change being quantified by measuring the difference in spectral absorbance (δ-absorbance) between 570nm and 600nm wavelengths of light. Cells from each line at the same passage stage were seeded in 6-wells of a 12-well plate (PDL-coated) at a concentration of 1x10^5 cells per well. A further 4 wells contained media but no cells to act as a baseline control. SC growth medium (1ml) containing 10% Alamar Blue™ was added to each cell line well on day 1 and the cells incubated at 37°C in 5%CO2 at 99% relative humidity. At 4, 8, 12, 24 and 48 hours, 50μl of medium was taken from each well (including the control wells), placed in a 96-well plate and assayed with a MRX-II plate reader spectrometer (Dynes Technology Inc, UK) to measure δ-absorbance: this absorbance is directly proportional to metabolic activity.

The metabolic activity of cells of rat of different age origins and at different passage number was also assessed using this method. Each cell type - SC, uMSC, dMSC, uASC and dASC - was grown in a 75 cm^2 flasks until reached the culture reached confluence. At this point the cells were trypsinised, counted and seeded into a new flask at the density of 5x10^5 cells. The passage number increased by one after each trypsination step; the proliferation assay was measured at passage 1 (P1), 5 (P5), 10 (P10), 15 (P15) and 20 (P20). Cells of each passage stage were seeded into a 12–well plate at a density of 2x10^3 cells per well; wells containing media only acted as a baseline control (Figure 3). Alamar Blue™ (10% v/v) was added to different wells at 24 h, 72 h, 96 h, 5 days, 7 days or 10 days and incubated for 14 hours. The
colour change was quantified by measuring the difference in spectral absorbance (δ-absorbance) at 570nm, 595nm and 600nm wavelengths of light for each time point. A MRX-II plate reader spectrometer was used to measure δ-absorbance, this value being directly proportional to the metabolic activity.

![Figure 3. Proliferation Assay description for each cells type at different time point](image)

**2.9 Cell growth curves**

A Schwann cell growth curve was created by plating cells were in a PDL-coated 24 well plate. Cells were plated in each well at a concentration 1x10^5 cells per well in 1ml of growth medium. The following day cells in 3 wells were trypsinised and the cells from individuals wells were counted using a Neubauer haemocytometer. This process was repeated each day for a further 8 days. Data was plotted and a best-fit growth curve constructed (GraphPad Prism, GraphPad Software, San Diego, California).

**2.10 Preparation of PHB conduits**

Poly-3-hydroxybutryate (PHB) conduits were made from sheets of sterile PHB (Astra-tech, Sweden) under aseptic conditions in a tissue culture cabinet. PHB sheets are manufactured by compressing two layers of PHB fibres together. The fibres of each layer of the PHB are unidirectional (Figure 4A) and individual layers are orientated so that when they are pressed together their fibres run perpendicular to the fibres of the adjacent sheet. It was therefore necessary to separate the layers before further use. To separate the layers, one corner of a sheet was gently lifted using a pair of jewellers forceps (Mercian, UK) to develop a gap/plane between the layers which were then slowly pulled apart (Figure 4B). One sheet is patterned from the pressing process and the other is smooth; the patterned sheet was discarded and the smooth sheet retained for fabrication of the conduits. To make a 1.4cm conduit used in the rat sciatic nerve gap model, a PHB sheet was cut into rectangles measuring 1.4cm x 0.8cm using a sterile scalpel blade. It is important that the direction of the fibres is parallel to the long axis.
of the conduit. The PHB sheet was then soaked in sterile water to prevent excessive melting during heat-sealing. The sheets were then wrapped around a 16G intravenous cannula and welded along the points where the sheets overlap using a fine-tipped soldering iron (Figure 4C and 4D). It is important that the smooth side of the sheet forms the inner wall of the conduit. The resulting conduit is 1.4cm in length with an internal diameter of 1.6mm which allows adequate space for post-injury swelling of the rat sciatic nerve. Each conduit was then placed in an individual well of a sterile 24-well plate for storage at room temperature.

Figure 4. (A) E. M. photomicrograph of PHB fibres and (B) splitting of PHB sheet into 2 layers. (C) Rolling of PHB sheet and (D) welding of conduit.
2.11 Operative procedures

2.11.1 Anaesthesia

All procedures were carried out in compliance with the regulations specified in the Animals (Scientific Procedures) Act 1986 (UK Home Office). Inbred adult male Sprague-Dawley rats (Harlan, UK; weights 250gms-300gms) were used in all experiments except for the last paper where Wistar rats were used. For recovery procedures animals were anaesthetised with isofluorane gas. The animal was placed in an induction chamber connected to an anaesthetic circuit. Oxygen was delivered at 2L/min following which 3% isofluorane was added for 1-2 min. After checking that most of the rat reflexes (pedal, palpebral, and corneal) were absent, the anaesthetised animal was then removed from the induction chamber and its left thigh was shaved and washed with alcoholic chlorhexidine. The animal was then placed on a sterile operating table. Anaesthesia was maintained by reconnecting the animal to the anaesthetic circuit via a face mask with oxygen flow rate set to 500ml/minute and isofluorane concentration set to 1.5%. Depth of anaesthesia was regularly assessed by monitoring the animal’s respiratory rate, heart rate and response to pain (tail pinch) with the isofluorane concentration being adjusted accordingly. Following the procedures for exposure, axotomisation and grafting (see section 2.11.2) of the sciatic nerve, muscle edges were re-opposed with a 4-0 vicryl suture (Johnson & Johnson Ltd, UK) and the skin was closed with a subcuticular Ethilon 6-0 suture. On completion of the operation 10µg of intramuscular analgesic buprenorphine (Vetergesic) was administered, the tail was marked with an indelible pen to identified each animals in the experimental group and the animal was placed on its right side on soft bedding in an individual cage. The animal was placed in a 35°C incubator and regularly monitored until it was fully conscious prior to re-colonisation. Animals were monitored daily for general health.

2.11.2 Gap repair using PHB conduit

To expose the sciatic nerve a 4 cm skin incision was made over the left gluteal region. A muscle splitting incision in the long axis of the fibres was then created in the biceps femoris to expose the sciatic nerve. Muscle and skin edges were retracted to allow adequate operative exposure. Under an operating microscope (Zeiss, X10 magnification) the nerve was gently mobilised and divided 5mm from the sciatic notch. For a 1cm gap a 1.4cm conduit was used, with each nerve end being placed 2mm into each end of the conduit thus leaving a 1cm gap between the nerve ends within the conduit. At each end the nerve was sutured to the conduit with a 9-0 epineural suture.
2.12 SC vitality and viability following retroviral transduction

The SC harvest and retroviral transduction with GFP were carried out according to the protocols set out in sections 2.1 and 2.5.2, respectively. Prior to transduction SC were split into 2 lines, one of which was transduced, while the other cell line acted as a control for vitality and viability measurements. Non-transduced SC (ntSC) and GFP transduced SC (GFP-SC) were cultured in identical conditions. Transduction efficiency was measured at 1 week and 6 weeks following transduction. As a measure of cell vitality cell growth curves for ntSC and GFP-SC were constructed 1 week following transduction (see section 2.6.2); cell viability was assayed using an Alamar Blue™ indicator solution (see section 2.8). *In vitro* fluorescent immunohistochemistry was performed to examine SC morphology.

Alginate hydrogel has been used extensively as a delivery vehicle for cultured Schwann cells (Mosahebi, Fuller et al. 2002) and growth factors (Mohanna, Terenghi et al. 2005). Alginate is a polysaccharide that was first described by the chemist E. C. C. Stanford in 1881. It is known to be one of the constituents of the structural component of marine brown algae. Alginate is a polymer of uronic acids, specifically β-D-mannuronic acid (M-alginate) and α-L-guluronic acid (G-alginate) (Figure 5). Alginate solidification occurs following the addition of a cationic buffer (e.g. Mg$^{2+}$ or Ca$^{2+}$), which creates crosslinks between polymer strands. The degree of alginate gelling is dependent upon the concentration of and length of exposure to the cations. Previous work has shown that for the purpose of supporting cells and growth factors in nerve conduits gaps a homogenous solutions of alginate is optimal (Mohanna, Terenghi et al. 2005). The degradation time frame of alginate within a mammalian system is about 2 years: the persistence of the polymer in mammalian systems is due to the lack of mammalian enzymes with the ability to catalyse breakage of the cross links between the polymer strands, and for that reason it could become a physical barrier to nerve regeneration. Although regeneration through alginate is seen at early time points, at long time points axonal regeneration is less vigorous. However, the addition of growth factors (for example, GGF) to alginate appears to significantly improve regeneration (Mohanna, Terenghi et al. 2005). Nevertheless, the use of alginate does have a number of advantages, such as it is commercially available in a highly pure endotoxin-free form (Pronova Biomedical, Oslo), which makes it suitable for use in tissue culture system. It is non-immunogenic and has been use for delivery of drugs *in vivo* and for cell transplantation (Duvivier-Kali, Omer et al. 2001).
2.12.1 *In vivo* study of GFP-SC transplanted in PHB conduits

Following injury to a peripheral nerve, Schwann cells play a vital role in promoting the regeneration of axons by providing both physical support and neurotrophic guidance. Fluorescent immunohistochemical techniques were used to assess nerve regeneration through bio-engineered conduits. Therefore, it was advantageous to label transplanted SC within these conduits with a fluorescent marker (GFP) that can be visualised by a combination of direct visualisation and using fluorochrome labelled antibodies (an anti-GFP antibody). Green fluorescent protein (GFP) (Shimomura, Johnson et al. 1962) is a bioluminescent protein that, when isolated and excited by blue light (absorbance peak 395nm), emits green light at an emission maximum of 508nm (Cubitt, Woollenweber et al. 1999). This study assessed the efficiency of labelling and cell vitality after labelling *in vitro*, and ease of direct identification of labelled cells *in vivo* and in combination with immunofluorescent histochemical protocols. GFP-SC were transplanted, within a PHB conduit, into a 1cm rat sciatic nerve gap model. Two experimental groups (the cellular group containing GFP-SC and an acellular, control group) were constructed (n=5) using adult male Sprague-Dawley rats. Conduits constructs were fabricated as described in section 2.9. In the cellular group, conduits were filled with GFP-SC suspended in 1.8% LVM alginate at a concentration of 80x10^6 cells/ml followed by gelling for 2min in 0.1M CaCl_2; the acellular group conduits contained only alginate gel. Anaesthetic and operative procedures were carried out according to the protocols described in section 2.10.1. Conduits were harvested after 2 weeks and processed for fluorescent immunohistochemistry for axonal and Schwann cell regeneration using anti-PGP (neuronal marker) and anti-S100 antibodies, respectively. Following conjugation with a Cy3-labelled (fluorescent) secondary antibody, sections were examined under a fluorescence microscope (Olympus BX60) and images captured using a cooled digital camera (Evolution QE).
The distance of penetration for the sprouting neurites into the alginate-filled conduit were quantified as a measure of axonal regeneration distance.

2.12.2 Tissue collection for immunostaining (nerve)

Animals were killed in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Animals were killed by CO₂ narcosis by placing the animal in a CO₂ chamber. The concentration of CO₂ was automatically increased until the animal had stopped making any respiratory effort for more than 1 minute. Termination was then confirmed by cervical dislocation. Following termination of the animal, the grafted conduit together with proximal and distal sections of nerve, were exposed by dissection under an operating microscope (Zeiss, X10 magnification). The sutures were removed to prevent tearing of the tissue during later cryo-sectioning. For immunohistochemistry, the conduit and nerve were removed, pinned out on plastic to prevent shrinkage and distortion and placed in Zamboni’s fixative overnight at 4°C. Over the following few days, the specimen was washed in PBS-sucrose until the solution became clear and the tissue had sunk to the bottom of the container (30ml universal container; Sterilin Ltd, UK). The specimen was then blocked in OCT (Raymond A Lamb-Laboratory Supplies, UK), with a piece of pre-fixed (in Zamboni’s solution) rat liver at the proximal end for orientation during microscopical examination and slowly frozen by carefully immersing in liquid nitrogen. The frozen block was stored at –40°C. For cryo-sectioning, a frozen specimen block was transferred to a cryostat (Hacker Bright Model OTF Cryostat, Bright, UK) and left for 10min to allow the temperature of the block to equilibrate with that of the cryostat. The block, in the appropriate orientation, was attached to the cryostat chuck using OCT (carefully frozen using a cryospray Bright, Raymond Lamb). The block was slowly trimmed back until the lumen of the conduit was reached. Longitudinal sections were cut so that proximal and distal nerve stumps were in-continuity. Cryosections were taken at 15μm thickness and placed on Vectabond™ (Vector Laboratories, UK) coated glass slides. The sections were allowed to dry overnight in a 37°C oven. The sections were either stained the day after cutting or stored wrapped in aluminium foil at –40°C for a maximum of 7 days.

2.13 Bone marrow stem cell transplantation

The transplantation of cultured Schwann cells into bio-engineered conduits has been shown to improve nerve regeneration in experimental models of peripheral nerve injury (Mosahebi, Simon et al. 2001).
However, the use of autologous cultured SC for the treatment of acute injuries may be impractical due to the technical difficulties and time required for harvesting and expanding such cells. The ideal ‘transplantable cell’ should be easily accessible, capable of rapid expansion in culture, immunologically inert, capable of long-term survival and integration in the host tissue, and amenable to stable transfection and expression of exogenous genes. Mesenchymal stem cells or marrow stem cells (MSC) have displayed unorthodox plasticity in their ability to trans-differentiate across what were once thought to be inaccessible oligolineage boundaries. When transplanted into models of injury, MSC have been reported to differentiate into a variety of cell types including retinal cells (Tomita, Adachi et al. 2002), astrocytes (Kopen, Prockop et al. 1999), hepatocytes (Lagasse, Connors et al. 2000), myocardium (Orlic, Kajstura et al. 2001) and myelinating cells of the peripheral nervous system (Dezawa, Takahashi et al. 2001). *In vitro* studies have shown that MSC will differentiate into cells expressing neuronal cell markers when exposed to neuronal cell mitogens, such as BDNF and NGF (Mahay, Terenghi et al. 2008). Glial growth factor (GGF) is a Schwann cell mitogen that has been shown to stimulate peripheral nerve regeneration. This study examined the effect of GGF on adult rat MSC morphology and phenotypical expression for glial marker like S-100 and p75 *in vitro*, and the effect of transplanting differentiated (dMSC) and undifferentiated marrow stem cells (uMSC) into a nerve gap conduit.

### 2.13.1 *In vitro* study

Schwann cells and marrow stem cells were harvested from isogenic laboratory bred Sprague-Dawley rats and cultured as described in section 2.2 and section 2.4, respectively. For identification by immunofluorescent and histochemical analysis, the cells were retrovirally transduced with GFP (see section 2.6.2). The MSC were differentiated towards a glial cell lineage by culturing in the presence of forskolin, PDGF, bFGF and GGF for 2 weeks (see section 2.5.2); MSC were also cultured in the absence of GGF to produce a line of undifferentiated cells (uMSC). The colony forming ability of freshly harvested MSC was measured as an indication of clonogenic capacity. The cells were further characterised by fluorescent immunocytochemical methods using anti-Thy 1.1 (a marker of undifferentiated MSC), S100 or GFAP antibodies, followed by incubation with secondary fluorochromes; Hoechst 33342 nuclear stain was used to identify cell nuclei. Following transfection with the MMLV-GFP retrovirus the transduction efficiency was measured as per section 2.5. The
stained, fluorescent cells were examined under a fluorescence microscope (Olympus BX60) and images captured using a cooled digital camera (Evolution QE).

2.13.2 In vivo study

The standardised PHB conduit-bridged 1cm nerve gap repair model described in section 2.11.2 was used to assess the abilities of MSC to substitute for Schwann cells during axonal regeneration. The PHB conduits contained intraluminal dissociated PHB fibres, which acted as a support matrix for the transplanted cells. There were 4 groups, each comprising five young adult Sprague-Dawley rats (n=5), which had the 1 cm nerve gap as bridged with the following constructs:

**Group 1:** Acellular control conduits (conduit alone)

**Group 2:** Conduits containing cultured Schwann cells (SC)

**Group 3:** Conduits containing undifferentiated marrow stem cells (uMSC)

**Group 4:** Conduits containing differentiated marrow stem cells (dMSC)

Under aseptic conditions, the conduits were constructed from a PHB sheets as described in section 2.10. Cells were suspended in DMEM (Invitrogen, UK) at 80x10⁶ cells/ml prior to seeding within the conduit. The anaesthetic and operative procedures were performed as described in section 2.10.1. Following grafting of the conduit, 30μl of cell suspension were injected into the lumen of the conduit, via the distal end, using a Hamilton syringe (Model 705, 50 μl): the needle was inserted until the tip of the needle reached the middle of the conduit then cell suspension very slowly injected. At 2 weeks post-grafting, the conduits, together with proximal and distal nerve ends, were harvested and placed into Zamboni’s fixative in preparation for immunohistochemical staining with S100 and PGP9.5 anti-sera. Sections were mounted in fluorescent mounting medium (Vectashield™, Vector Lab, UK) and examined under using a fluorescence microscope (Olympus BX 60) with a blue filter to identify GFP-labelled cells. Schwann cell presence and location using immunostaining for anti-S100 antibody and axonal regeneration distances (mm) using anti-PGP antibody were measured at low magnification with a graticule. ANOVA and a Tukey’s test were applied using statistical analysis software (SPSS Inc.).
2.14 Myelin formation

2.14.1 Primary sensory neuronal cultures

Adult sensory neuron culture was first established by Scott in 1977 (Scott 1977) and later adapted by Lindsay (Acheson and Lindsay 1996). The method described below has been optimised for 40-45 ganglia, which is the number of ganglia harvested from one adult rat. All incubation steps took place in temperature controlled chambers with 95% relative humidity at 37°C in 5% CO₂ unless otherwise stated. Adult male Sprague-Dawley rats (Harlan Sprague Dawley Inc, UK) were killed as described in section 2.12.2, their vertebral columns were removed and cut in to expose the dorsal root ganglia. These were then gently pulled from the ganglia cavities using fine forceps, and collected into a sterile culture dish (Corning Life Sciences®) containing F-12 Nutrient Mixture (Invitrogen, UK) 1x with L-glutamine supplemented with 0.5% (v/v) penicillin/streptomycin at 37°C. To remove nerve roots and capsular connective tissue each ganglion was cleaned under a low power dissecting microscope (Meiji EMZ, Meiji Techno Co., Japan) using a scalpel blade and the ganglia collected into a sterile 35mm culture dish containing 1.8ml F-12 nutrient medium.

Dissociation of the ganglia was accomplished by gentle one hour enzymatic digestion using 0.125% (v/v) collagenase type IV (Worthington, UK) in F-12 nutrient medium: this step was repeated twice more. After removing medium and repeating the washes 2 times with F12, 0.2% (v/v) trypsin in F12 (Worthington, UK) was added to the ganglia and incubated for 30min. The medium was then removed and 30% (v/v) foetal bovine serum (Invitrogen, UK) in F-12 was used to inactivate the trypsin. The ganglia were washed and then mechanically dissociated by gentle trituration using a glass Pasteur pipette. The single cell suspension obtained was passed through a 70µm filter (BD Falcon, UK) to remove clumps of cells and myelin. The suspension was centrifuged (Centaur 2, MSE) at 200xg for 5min and the medium aspirated from the pellet to remove dead cells and debris. Following centrifugation, all but the last 500µl of the supernatant was removed from the cell bolus. The cells were re-suspended in the remaining supernatant fluid and gently layered over 15% (v/v) bovine serum albumin (BSA, Sigma, UK) in F-12 and centrifuged at 200xg for 10min. All supernatant fluid and cell debris were gently removed to leave the bolus of dissociated neurons. The neurons were re-suspended in 1ml modified Bottenstein and Sato’s medium, which comprises 0. 1mg/ml transferrin, 20nM progesterone, 100µM putrescine, 30nM sodium selenite, 1mg/ml BSA, 0.01mM cytosine arabinoside and 10pM insulin (all Sigma-Aldrich) in F12 medium. The neurons were plated into two pre-coated 6-well plates (BD Falcon, UK); the pre-coated 6 well plates were prepared as follows. The first coating of poly-D-ornithine 0. 5mg/ml (Sigma)
was applied the day before and incubated overnight at 4°C. The day after the excess of solution was aspirated off and after two washed with F12 the plates were to be used. Next, laminin-1 (2µg/ml, Sigma-Aldrich) was applied to the wells and incubated for 2h at 37°C, 5% CO₂. The plate was then washed twice with F12 only, before adding the neurons into the wells.

Two hours after neuron seeding, 150ng/ml nerve growth factor (NGF2.5, from mouse submaxillary glands, Sigma, UK) was added to each culture to induce the DRG neurons to develop neurites: the NGF was reconstituted in F-12 containing 1. 0mg/ml BSA (Sigma, UK) to a stock concentration of 10µg/ml, which was then further diluted with F-12 to give final required concentration.

2.14.2 Cell-DRG contact co-culture
For the myelination studies, dissociated DRG neurons were cultured for 3 days the dMSC, dASC or SC were seeded directly into the well containing the cultured neurons. The co-culture were then maintained in medium composed of 50% of either dMSC, dASC differentiated medium or SC medium enriched with the growth factors (GGF, PDGF-AA and bFGF) plus 50% of BS neuron culture medium. After 24h co-culture, 100µg/ml brain derived neurotrophic factor (BDNF, Autogen Bioclear, UK) and 50µg/ml ascorbate (Sigma) were added to the co-cultures and left to activate the myelination process for 14 days. At this end time-point, total RNA was extracted from one plate of co-cultured cells and proteins harvested from the other as described in 2.15 and 2.16. To investigate the effects Notch signalling on myelin expression another set of co-cultures were set up as described above. Differentiated ASC were seeded over the DRG neurons in the absence or presence of the Notch inhibitor DAPT (25µM). After 24 h, 100µg/ml brain derived neurotrophic factor and 50µg/ml ascorbate were added to the cultures and left to activate the myelination process for 14 days. At the end of the incubation period total RNA was extracted from the cells and the levels of protein zero (P0) and peripheral myelin protein-22 (PMP-22) transcripts assessed.

2.14.3 Cell-DRG non-contact co-culture
DRG neurons were cultured in 24-well plate on laminin-coated 15mm diameter glass coverslips (Scientific Laboratories Supplies, UK) for 24 hour. SC, dMSC or dASC from rats of different ages (neonatal, young and old) were seeded at a density of 42 000 cells (per insert) onto 0.1µm pore-size 24-well plate inserts (SLS, UK).
The inserts plus cells were cultured in cell growth medium plus the required growth factors at 37°C and 5% CO₂ for 24 hours. After 24 hours the co-cultures were set up by gently placing the pre-seeded inserts into the wells containing DRG. In separate wells containing DRG cultures, but without transwell inserts, NGF was added (100µg/ml⁻¹) as a positive control. The negative control comprised DRG cultured in normal BS medium with an overlying insert containing only the medium used to culture the cells under investigation. The co-culture was maintained for 24 hours. DRG and the cell in the inserts were then fixed in 4% (w/v) PFA. DRG were stained with anti-βIII tubulin for analysis of neurite outgrowth.

2.14.4 NG108-15 contact co-culture
NG108-15 (EACC catalogue number 88112302, Porton Down, Wiltshire, UK) cell are a mouse neuroblastoma and rat glioma hybrid cell line which mimic motor neurons (Ling et al, 2005). NG108 cells were cultured in NG growth media comprising Dulbecco’s Modified Eagle Medium (DMEM, 5g glucose), 10% (v/v) FBS 1% (v/v) penicillin/streptomycin (Invitrogen, UK). For neuron co-culture experiments, differentiated ASC were plated at a density of 10 000 cells per well and allowed to settle for 24 h. Prior to seeding, the dASC cultures treated with DAPT NOTCH inhibitor were washed extensively to eliminate possible direct effects of the drug on neurite outgrowth. NG108-15 neurons were then added to the dASC monolayer at a density of 1000 cells in chamber slide-flask (Lab-Tek™, Thermoscientific, UK) and the co-cultures maintained for a further 24 h at 37°C in 5% CO₂. Cells were then fixed in 4% (w/v) PFA and washed twice with PBS before immunostaining for neurite outgrowth (see section 2.17.2).

2.15 Qualitative assessment of transcript levels of protein markers
2.15.1 Preparation of total RNA
Total RNA was isolated from each of the contact co-cultures (DRG-SC, DRG-dMSC and DRG-dASC). Following two weeks of co-culture, the cells were trypsinised, collected and centrifuge at 200xg for 5min; the supernatant was removed and the cell pellet washed in sterile PBS and centrifuged again at 200xg for 5min. The supernatant was discarded and the cells kept on ice. RNA was also isolated from cultures of SC, uMSC, dMSC, uASC and dASC obtained from rats of different ages (neonatal, young and old). The cells were cultured in a 75cm² flasks until they reached confluence (passage 7).
After trypsinisation the cells were washed in sterile PBS, centrifuged as above and the pellet washed in PBS.

For the Notch studies, total RNA from ASC cultures at key stages of differentiation - day 4 following retinoic acid treatment, day 5 following 24 h stimulation with glial growth factors and after 2 weeks of differentiation - was analyzed. RNA extraction for all cells was done using the RNeasy (Qiagen, UK) mini kit. Total RNA was isolated from the cell pellet comprising a maximum of 4x10^6 cells. The cell pellet was resuspended in RLT buffer with 0.01% (v/v) β-mercaptoethanol and then transferred into a QIAshredder spin column (Qiagen, UK) and centrifuged at 15.6xg for 2 min (GeneFuge 24D microcentrifuge, Progen Scientific, UK). The eluate was retained and mixed with 70% (v/v) ethanol. The solution was pipetted into an RNeasy mini column and centrifuged at 15.6xg for 15 sec. The column was washed through with RW1 buffer by centrifugation as described above. A DNase I digestion was performed whilst the nucleic acid was bound to the column. An aliquot of DNase I solution (Qiagen) was prepared following manufacturer’s instructions, pipetted onto the column membrane and allowed to incubate at room temperature for 15 min. The column was then washed with RW1 buffer and RPE buffer as specified in the manufacturer’s instructions. A final pre-elution centrifugation step was included to ensure that the complete removal of RPE buffer and to dry the silica membrane. The RNA was eluted by pipetting 50µl of RNase-free water onto the column membrane followed by centrifugation at 15.6xg for 1min and collection in an RNase-free micro tube (Starlab, UK). Total RNA was extracted from whole sciatic nerve was extracted using the same protocol and used as a positive control.

2.15.2 Determination of stock RNA concentration
The RNA concentration in each preparation was determined by UV absorbance spectrophotometry (UV1101 Biotech photometer, WPA, UK) at wavelength of 260nm over a path length of 1cm. One absorbance unit at 260nm corresponds to a concentration of 40µgml⁻¹ total RNA. The purity of the RNA was determined by comparing the absorbance of a given RNA solution at wavelength of 260nm and 280nm, then calculating the ratio A_{260nm}/A_{280nm}: a protein free RNA solution should have a ratio of 2.0.

2.15.3 Myelin and Notch primers
Oligonucleotide primers (Invitrogen, UK) were used to amplify the transcript of P0, MBP, and PMP22 by one-step reverse transcriptase-polymerase chain reaction (RT-PCR). Each
primer was designed such that forward and reverse primers anneal to different exons to eliminate the possibility of amplification of contaminating traces of genomic DNA. To study NOTCH expression, primers (Sigma, UK) were used to amplify the transcripts of NOTCH-1, NOTCH-2, Jagged-1, Delta-1, Hes-1, and Hey-1 by RT-PCR; the β-actin and HPRT transcripts were used as housekeeping genes (Table 1). In papers III and V the β-actin transcript was used to verify the integrity of the templates RNA for amplification and to act as normalising reference gene transcripts. The optimum annealing temperature for each pair of primers was determined by experiment.

2.15.4 Reverse transcriptase–polymerase chain reaction (RT-PCR)

The Qiagen One-Step RT-PCR kit (Qiagen Ltd, UK) was used for all RT-PCR. A master mix was prepared in RNase-free water to give the final concentration of components: 1x Qiagen OneStep RT-PCR Buffer, 400µM of each dNTPs, 0.6µM of forward primer, 0.6µM of reverse primers, 5-10 units of Qiagen OneStep Enzyme mix and 1ng template RNA or RNase-free water (no template control, NTC). An MJ Research PTC-200 (gradient) (MJ Research Inc, USA) thermal cycler was used for all reactions. The cycling parameters were as follow: a reverse transcription step of 50°C for 30min followed by a nucleic acid denaturation/reverse transcriptase inactivation step of 95°C for 15min then 35 cycles of denaturation (95°C, 30sec), annealing (65°C, 30sec) and primer extension (72°C, 1min) followed by final extension incubation (72°C, 5min). The amplification of the β-actin and HPRT house-keeping gene transcripts were done to access the efficacy of the RNA as a template for reverse transcription and subsequent amplification. Qualitative assessment of the quantity and linear size of the amplicons was determined by 2% (w/v) agarose (Melford Laboratories, UK) gel electrophoresis. Each agarose gel was soaked in 0.1% GelRed solution (Sigma) solution for 10min. The gel was then rinsed in water and images were captured using an AlphaImager 2200 (AlphaInnotech, USA) gel documentation system with UV transillumination.
Table 1. Oligonucleotide primer pairs used for RT-PCR amplification of the transcripts of interest.

<table>
<thead>
<tr>
<th>Myelin Proteins</th>
<th>Forward (5’- 3’)</th>
<th>Reverse (5’- 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Zero (P0)</td>
<td>GGTGGTGCTGTTGCTGCTG</td>
<td>TTCGTGCTTCCGGCTGCTGGTC</td>
<td>(Lemke and Axel 1985)</td>
</tr>
<tr>
<td>Myelin Basic Protein (MBP)</td>
<td>TTCTTTAGCGGTGACAG</td>
<td>CTGTCTCTTTCTCCCCA</td>
<td>(Roach, Boylan et al. 1983)</td>
</tr>
<tr>
<td>Peripheral Myelin Protein (PMP22)</td>
<td>CTGTACCACATCCGCTTGG</td>
<td>TCAACACGAGGCTGACGGTC</td>
<td>(Welcher, Suter et al. 1991)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CACCACAGCTGAGGGGAAATCGTGCGTGA</td>
<td>ATTTGCGGTGCACGATGGAGGGACTC</td>
<td>(Segovia, Lawless et al. 1994)</td>
</tr>
<tr>
<td>NOTCH-1</td>
<td>CTTGTGAAAAATGACGCCC</td>
<td>CCTTATTGCCTGCATCCTCCT</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>NOTCH-2</td>
<td>TTTGCTGTGGAAGACGACC</td>
<td>GCCCCATGTTGCTCTGGGCGT</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>JAGGED-1</td>
<td>ATGGCCTCAACGATACTCCT</td>
<td>ACATGTACCCCCATAGTGGCA</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>DELTA-1</td>
<td>CACGAGAAAACCAGAAGC</td>
<td>ATGCCCGGAAGTCTATGTG</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>HES-1</td>
<td>GTCCCCGGGTGGCTGCTAC</td>
<td>AACACGCTCGGCTCTGTGTGCT</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>HEY-1</td>
<td>AAGACGGAGGCACTCATCG</td>
<td>GCAGTGTGCAACATTTCCC</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
<tr>
<td>HPRT</td>
<td>CAGGCCAGCTTTTGGTGAT</td>
<td>TCACTTTTCGCTGATGACAC</td>
<td>(Rooman, De Medts et al. 2006)</td>
</tr>
</tbody>
</table>
2.15.5 Sequencing of PCR amplicon: QIAquick gel extraction
The sequences of the amplicons were verified by the dideoxy chain termination DNA sequencing methodology (Sanger et al., 1977). The individual amplicons were isolated from agarose gel slices and purified using the QIAquick gel extraction protocol and kit (QIAGEN) according to manufacturer’s protocol. The gel slices were excised under UV transillumination using a sterile scalpel blade for each band and placed into pre-weighted microtubes tubes. Following addition of the Q-buffer, an aliquot of 10µl of 3M sodium acetate, pH 4.5 (S7899, Sigma, UK) was added to ensure that the correct (acidic) pH was obtained. The amplicons were eluted from the purification column into double distilled water and were stored at -40°C until required.

2.15.6 Big Dye™ Terminator Sequencing
The PCR amplicons sequenced using the BigDye® Terminator 3.1 Cycle Sequencing (Applied BioSystems, Foster City, USA) chemistry in accordance with the manufacturer’s protocol. Amplicons were sequenced in the forward and reverse directions to ensure to complete sequence determination and accuracy. In brief, for one reaction the following reagents were combined in a 0.5ml microtube tube: 4.0µl Terminator ReadiMix, 1.0µl of 1.6µM primer and 5.0µl (5-20ng) of the PCR amplicon in water and made to a final volume of 10µl with tissue culture grade water (Sigma, UK) in a microfuge tube. The components were mixed and centrifuged briefly in a microfuge then placed in a thermal cycler (MJ PTC-200). The following cycling parameters were used: one cycle at 96°C for 4min, 25 cycles at 96°C for 30sec; 50°C for 15 sec ramping at 1°C per second and 60°C for 4min ramping at 1°C per second and final constant incubation at 4°C. After the reactions completed, the dye terminators were removed by ethanol precipitation. Briefly, the sequencing reactions were made up to a volume of 100 µl with tissue culture grade water and transferred to fresh microtubes. Aliquots of 10 µl of 3M sodium acetate, pH 4.5, 1 µl of GlycoBlue (Applied BioSystems, 9515) and 250 µl of 100% ethanol were added and the final solution mixed. The mixtures were placed on ice for 10 min then centrifuged at 10 000xg for 15min. The supernatant fluid was discarded and 250µl of 70% ethanol added. The tube was inverted a couple of times to allow the pellet to dislodge then the pellet collected by centrifugation; the wash step was repeated once more. After the second wash, the supernatant was removed completely and the pellet allowed to dry at ambient temperature for 20min then incubated at 90°C for 2min to remove any remaining traces of ethanol.
The dried pellets were taken to the University of Manchester DNA sequencing facility for capillary electrophoresis and base-calling on a 16 capillary ABI Prism 3100 Genetic Analyzer.

2.16 Protein Assay
A set of BSA (Sigma, UK) protein standards (0.25mg, 0.5mg and 1mg ml⁻¹) were prepared in lysis buffer (100mM Pipes, 5mM MgCl, 20% glycerol (v/v), 0.5% (v/v) Triton-X, 5mM EGTA and 0.005% (v/v) protease inhibitors (Sigma). The protein assay reagents were added to the standards and samples (diluted 1:20) using the DC protein assay system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. This resulted in coloured reaction products. The absorbance of the standards and samples were read using the MRX spectrophotometer (Dynex, Tech., UK) at 750nm. The absorbance was read using the MRX spectrophotometer and the readings were used to construct a standard curve of absorbance versus protein concentration using GraphPad Prism to calculate the concentration of the proteins in the samples.

To study the expression of myelin proteins, SC, MSC and ASC cells (culture with or without DRG neurons) were scraped into 150μl lysis buffer and collected in microtubes (Starlabs, UK) and incubated on ice for 15min then centrifuged at 600xg for 5min. Samples of the whole brain and whole sciatic nerve were also collected and extracted using the same procedure for use as controls for the western blotting analysis. Protein samples from the different cell types (SC, uMSC, dMSC, uASC and dASC) from rats at different age (neonatal, young and old) were also collected. For Notch studies, ASC were differentiated in the presence or absence of DAPT notch inhibitor for a period of 2 weeks and the proteins extracted as described above.

2.16.1 Western blotting analysis for myelin proteins and glial marker
Polyacrylamide gels were cast and run using BioRad™ mini-gel apparatus (Mini-Protean® 3 Cell vertical electrophoresis system, Biorad, USA). Fifteen percent (w/v) polyacrylamide gels were used to resolve proteins MBP, PMP22, P0 and MAG. Whole cell protein extracts were diluted in Laemmli buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 0.125M Tris HCl, all Sigma) and heated to 95°C for 5min. Equal amounts of protein (10μg per well) were loaded onto the stacking gel and electrophoresed at 120V for 1hr 30min.
The separated proteins were transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, UK) at 80V for 90min in transfer buffer containing 25mM Tris-base, 192mM glycine, 20% (v/v) methanol (Fisher Scientific Ltd UK). Transfer of proteins was confirmed by brief staining with 10% (v/v) Ponceau red (Sigma) in 0.2% (v/v) in acetic acid. The membranes were gently agitated for 2hours in blocking buffer containing 5% (w/v) dry milk (Marvel, Premier International Foods, UK) in TBS Tween (10mM Tris pH 7.5, 100mM NaCl, 0.1% (v/v) Tween-20, all Sigma) and incubated with primary antibody for myelin proteins (anti-MBP mouse monoclonal, 1:1000, Serotec, UK; anti-MAG mouse monoclonal, 1:1000, Chemicon, UK; anti-PMP-22 goat polyclonal, 1:500, Santa-Cruz, UK or anti-P0 goat polyclonal 1:500 Santa-Cruz Biotech., UK) overnight at 4ºC. The following day the membranes were washed eight times for 5min using TBS Tween. The membranes were then incubated for 1hr with HRP-conjugated secondary antibodies as follows: horse anti-mouse (Sigma) for MBP and MAG and horse anti-goat 1:1000 (Sigma) for P0 and PMP-22. After a further eight washes with TBS Tween, the membranes were treated with ECL chemilluminescent substrate (Amersham, UK) for 1min and exposed to Kodak X-OMAT light sensitive film (supplied by Sigma, UK).

For ASC analysis following differentiation into SC-like cells, protein samples were resolved with 15% sodium dodecyl sulphate–polyacrylamide gels, transferred to nitrocellulose and the membranes were incubated overnight at 4ºC with either mouse anti-GFAP (1:200; LabVison, USA) or mouse anti-S100 (1:750; Chemicon, UK) antibodies. The following day the membranes were washed, incubated for 1hr with specific HRP-conjugated secondary antibodies and treated with ECL chemilluminescent substrate and exposed to Kodak XOMAT light sensitive film as described above.

2.16.2 Western blotting for protein-38 and protein-53

Equal amounts of protein (10μg per well) isolated from SC, uMSC, dMSC, uASC and dASC from animals at three different ages (neonatal, young adult and old) electrophoresed through a polyacrylamide gel at 120V for 1hr 30min as described above. The separated proteins were transferred to Hybond ECL nitrocellulose membranes and the transfer was confirmed by staining with Ponceau red. The membranes were gently agitated for 2 hours in blocking buffer and incubated with primary antibody (anti −p38α mouse monoclonal, 1:2000, BD Biosciences, UK; anti −p53 mouse monoclonal antibody, 1:250, Abcam, UK) overnight at 4ºC.
The following day the membrane were washed and incubated for 1hr with HRP-conjugated secondary antibodies. Finally the membranes were treated with ECL chemilluminescent substrate for 1min and exposed to Kodak XOMAT light sensitive film, as above.

2.17 Immunocytochemistry

2.17.1 Cells staining

The three types of co-cultures, DRG-dMSC, DRG-SC and DRG-dASC, were incubated for two weeks in slide flasks (Fisher Scientific Ltd, UK) with the same type of medium described previously, then fixed in 4% (w/v) PFA at room temperature for 30min. The cells were blocked for 1hr in a solution of normal rabbit serum (1:100, Dako, UK) and normal goat serum (1:100, Sigma) in antibody diluent (0.03% (v/v) Triton X-100, 0.10% (w/v) Bovine Serum Albumin, 0.10% (w/v) sodium azide, all Sigma) and incubated overnight at 4°C with primary antibodies for P0 (1:50, goat polyclonal, Santa Cruz, USA) and βIII-tubulin (1:500, mouse monoclonal, Abcam). The following day, the cells were incubated at 21°C for 2h) with appropriate secondary antibodies: FITC-conjugated rabbit anti-goat (1:100, Vector Laboratories, UK) for P0 and Cy3-conjugated mouse anti-goat (1:200, GE Healthcare, UK) for βIII-tubulin.

Differentiated ASC were fixed as above and incubated with mouse anti-glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA) and rabbit anti-S100 (1:500; Dako, Denmark) overnight at 4°C and then incubated with Cy3 goat anti-mouse (1:200; Amersham, UK) or FITC goat anti-rabbit (1:100; Vector Labs, UK) conjugated secondary antibodies (25°C, 2hours). The uMSC and uASC from neonatal, young and old rats were prepared and fixed as described above. After the fixation and permeabilization steps, the cells were blocked for 1hr in a solution of normal horse serum (1:100, Sigma, UK) in antibody diluent and incubated overnight with primary antibody against Stro-1 (1:50, mouse monoclonal, R&D, UK) at 4°C. The following day, the cells were incubated at room temperature for 2 hours with the secondary antibody FITC-conjugated horse anti-mouse (1:100, Vector Laboratories, UK).

All preparations were mounted using Vectashield™ for fluorescence with DAPI (Vector Laboratories, UK) and examined under an Olympus BX60 fluorescence microscope. Images were captured at x20 magnification with an Evolution™ QE digital camera and the appropriate fluorescence filter.
2.17.2 NG108-15 staining
NG108-15 neurite outgrowth on cultured ASC was visualized using mouse anti-neurofilament protein antibody (1:500; Abcam, UK) as previously described. The secondary antibody, goat anti-mouse cyanine-3 (Amersham Bioscience, UK), at 1/200 dilution was added and incubated for 1 hour at room temperature. The cells were washed 3 times for 5 min with PBS and the slides mounted using anti-fading Vectashield solution (Vector Labs, UK). The average neurite length and neurite number per NG108-15 cell was determined from inspection of 100 NG108-15 cells for each condition in each experiment (n = 3). Kruskal–Wallis one-way ANOVA with Dunn’s comparison test was used to determine the statistical significance between data sets.

2.17.3 Dorsal root ganglia staining
DRG neurons were stained for the neuronal cytoskeletal markers βIII-tubulin (1:500, mouse monoclonal, Abcam). The following day, the cells were incubated (21°C, 2h) with secondary antibodies FITC-conjugated horse anti-mouse (1:100, Vector Laboratories, UK). DRG were assessed for neurites outgrowth following transwell co-culture. For each co-culture type neurites outgrowth was assessed on 15 random fields and the length of the longest neurite was calculated by tracing its length from the most distal point back to the cell body using ImageJ programme. Analysis was carried out using GraphPad Prism 4 programme and the statistical test one-way ANOVA was applied with Bonferroni post-test.

2.17.4 Mitochondrial-specific staining
uMSC, dMSC, uASC and dASC (from neonatal and old rats) were stained with MitoTracker (MtT), Red (M7512, CMXRos, Invitrogen) and βIII-tubulin (1:2000 rabbit polyclonal, Abcam, UK). The cells were plated at a density of 1 x10^4 in a chamber-slide flask and let them grow for 24h. The day after they were washed with sterile PBS and the medium was replaced with 1mL of MitoTracker solution (2% BSA + MtT Red, diluted 1:20 000) and incubated for 20 min at room temperature. The cells were then washed twice using sterile PBS and incubated with 4% (w/v) PFA/PBS solution for 30 min at RT. Finally, they were stained for βIII-tubulin by immunocytochemistry as described above.

2.18 Scanning electron microscopy analysis (SEM)
For SEM studies, DRG co-cultured with SC, dMSC or dASC were established as described above and kept in culture for two weeks. The samples were then fixed with 2.5%(v/v)
glutaraldehyde (Sigma, UK) in phosphate buffer (0.1M, pH 7.4 at 4°C x 30min). Subsequently, the samples were dehydrated in a graded series of ethanol solutions (50%, 75%, 100%) and coated with silver particles prior to examination with a scanning electron microscope (SEM Philips 52M).

2.19 Transmission Electron Microscopy (TEM)
Samples of SC, uMSC and uASC from young and old rats were fixed in a solution of 2.5% (v/v) purified glutaraldehyde (Sigma, UK) and 0.5% (w/v) saccharose (Merck, Darmstadt, Germany) in 0.1M Sörensen phosphate buffer (0.2M Sodium Phosphate, dibasic; 0.2M Potassium Phosphate, monobasic) pH 7.4, for 6–8 h, then washed and stored in 0.1M Sörensen phosphate buffer with addition of 1.5% (w/v) saccharose at 4–6 °C prior to embedding.
Before embedding, the samples were washed for few minutes in Sörensen phosphate buffer and then immersed in 2% (w/v) osmium tetroxide (Sigma) in the same buffer solution for 2 h. The samples were carefully dehydrated through graded alcohols series with at least five times of 5 min each. After two passages (7 min each) in propylene oxide (Sigma) and 2 h in a 1:1 mixture of propylene oxide and Glauerts’ mixture of resins, the cells were embedded in Glauerts’ mixture of resins, which comprises equal parts of Araldite M and the Araldite Harter, HY 964 (Merck) with the addition of 2% (v/v) accelerator 964, DY 064 (Merck) with the addition of a plasticizer (0.5% (w/v) of dibutylphthalate) that promote the polymerization of the embedding mixture. For the final step, a 2% accelerator 964, DY064 (Merck) was added. Thin sections of the samples were cut in a thickness range of 50-70 nm with an ultramicrotome (Ultracut UCT, Leica Microsystems). Sections were collected and placed on grids previously coated with pioloform film. For transmission electron microscope, grids were stained with uranyl acetate (saturated solution) for 15 min and lead citrate for 7 min, then washed and dried.
3.0 Results

3.1 *In vitro* studies (Paper I)

Cultured SC displayed classical spindle-shaped morphology on immunostaining (Figure 1A, paper I). SC cultures transduced with GFP (GFP-SC) were found to exhibit bright green fluorescence in vitro (Figure 1B, paper I). The number of cells expressing GFP as a percentage of the total number of cells in culture (transduction efficiency) was 39.4% at week 1: this value being maintained at 37.9% up to week 6. This result indicates that the transduced cells were stably transfected and that they continued to divide generating further cells expressing GFP without significant dilution of transgene expression. The intensity of fluorescence did not qualitatively deteriorate between week 1 and week 6. When monitoring GFP-SC cultures, it was found that removing culture medium and replacing with pre-warmed (37°C) sterile phosphate buffered saline (PBS) reduced the background auto-fluorescence seen under the microscope caused by components of the culture medium. The PBS was replaced with cell culture medium prior to returning the cells to the incubator.

An Alamar Blue™ assay was used to measure the metabolic activity of cultured cells. Data were collected over a period of 48h and linear regression analysis applied to the values obtained; there was no significant difference was found between the gradients of the GFP-SC and ntSC graphs indicating comparable metabolic rates. Growth curves were constructed comparing the growth of neonatal SC (ntSC) and GFP-SC over a 7 day period in vitro (Figure 3, paper I). The exponential phase of growth began at day 3 and day 4 in the ntSC and GFP-SC cultures, respectively; the plateau (cell confluence) phase of growth was reached at day 6 and day 7 in ntSC and in GFP-SC, respectively. At confluence, the ntSC and GFP-SC cultures reached a comparable cell number (9.05 x 10^5 cells/ml and 8.4 x 10^5 cells/ml, respectively on a 75cm² flasks). These measurements of vitality and growth have shown that GFP-SC are slower to establish themselves in culture following passage, but following this initial period they achieve growth rate profiles comparable to those of equivalent non-transduced cells.

3.1.1 *In vivo* studies GFP fluorescence on histological analysis

At x40 magnification transduced GFP-SC cells were easily identifiable on histological sections under blue ultraviolet fluorescence light. Staining with an S100-Cy3 (red) conjugated antibody showed co-localisation of red and green fluorescence and GFP-SC (Figure 4, paper I). As expected at this early time point GFP-SC were located evenly throughout the length of the conduit, as cells were initially transplanted in a uniform suspension of alginate. GFP-SC
in the proximal stump of the conduit were found exhibiting extensive bipolar cytoplasmic processes in close association with regenerating axonal fibres indicating integration in the regenerative process. When taken together with the significant improvement in the regeneration distances this indicates GFP-SC were contributing to the regenerative process. The inherent fluorescence of GFP-labelled cells greatly simplified the staining procedure required to analyse sections. GFP-SC were incapable of producing functional GFP-containing retrovirus, therefore it can be assumed that visible cell labelling was restricted to transplanted cells with no contamination of the surrounding tissues, as is frequently seen with chemical labelling methods.

3.1.2 Nerve regeneration
Regeneration was assessed by measuring axonal (PGP-staining) and Schwann cell (S100-staining) regeneration distances from the proximal stumps and through the conduits. The transplantation of GFP-SC significantly improved the rate of nerve regeneration in comparison with the control group (Figure 5, paper I). Both axonal and Schwann cell regeneration distances were significantly better in the GFP-SC groups (3.2±0.45 vs. 2.4±0.14mm; \(P<0.05\) and 3.55±0.62 vs. 2.55±0.21mm; \(P<0.05\) respectively; paired t-test).

Comparison of the effect of GFP-SC transplants on nerve regeneration in this study with those from previous studies, which used lacZ-SC transplants in the same model of nerve injury, in this laboratory showed that there is an improvement in the use of GFP compared to lacZ labelling methods for SC tracking within conduits. This indicates that GFP transfection had no detrimental effect on SC physiology in vivo in comparison with lacZ labelling.

3.2 Marrow stem cell culture and phenotypical characterisation (Paper II)
Mesenchymal stem cells (MSC) were collected by plating and culturing bone marrow aspirate. Freshly harvested MSC showed a high clonogenic ability, which increased with plating density and approached ~13%. MSC grew rapidly in the presence of basic growth factors with a doubling time of 48h. Prior to transplantation within PHB conduits, MSC were transfected with the GFP carrying retrovirus; the transduction efficiency was high as a result of the high mitotic rate of MSC and reached up to 80% cell transduction/GFP expression. Following two weeks exposure to GGF the MSC were found to express the glial cell markers S100 and GFAP and approximately 10% of these cells displayed the classical bipolar, spindle-shaped morphology of native SC. Thus, within the MSC population exposed to GGF, there were cells displaying glial cell phenotypical and morphological characteristics.
3.2.1 *In vivo* results of transplanted GFP-dMSC

Immunohistochemical analysis of the conduits containing uMSC and dMSC identified transplanted cells by their expression of GFP: dMSC were found to be distributed evenly throughout the lumen of the conduit (Figure 1, paper II). At the proximal end of the conduit dMSC were found to be adherent to intraluminal PHB fibres (Figure 1A and 1B, paper II), and to be directly involved with axonal and Schwann cell regeneration (Figure 1A and 1C, respectively, paper II). In conduits containing uMSC, a small number of transplanted cells were detected by their GFP fluorescence at the proximal and distal ends of the conduit and they were found to express S100 (Figure 1D, paper II), which indicated possible effects of local cellular and humoral factors on marrow stem cell differentiation. Axonal regeneration distance (PGP; Table 1, paper II) was significantly better in the SC seeded conduits in comparison to the control (empty), uMSC- and dMSC-seeded conduits. The uMSC and dMSC seeded transplants conferred some beneficial effect on axonal regeneration compared to the cell-free control, but this was not a statistically significant effect. Schwann cell regeneration distance (S100; Table 1, paper II) was significantly better in the Schwann cell and dMSC transplanted groups compared to the cell-free (control) and uMSC groups. From these results it can be concluded that marrow stem cells previously exposed to GGF and expressing glial cell markers prior to transplantation appear contribute to increased Schwann cell regeneration.

3.3 Myelin Protein Transcripts (Paper III)

RT-PCR showed transcripts for P0 (190bp amplicon), MBP (230bp and 306bp amplicons) and PMP-22 (636bp amplicon) to be present in both DRG-dMSC and DRG-dASC co-cultures and in the DRG-SC co-culture (positive control) (Figure 1, paper III). RT-PCR amplification efficacy of mRNA was confirmed by amplification of the β-actin (510bp amplicon) housekeeping gene transcript, which also acted as a reference gene. Individual cultures of DRG, SC, dMSC and dASC alone were also analysed. SC expressed the transcript for P0; whereas DRG, dMSC and dASC did not (Figure 1, paper III). Also, DRG neurons cultured alone (negative control), but using the same conditioned medium as for co-cultures, did not express transcripts for any myelin proteins. SC, dMSC and dASC expressed the PMP22 transcript. MBP transcripts were present in isolated cultures of dMSC and SC, but not in dASC cultures alone. When present, the MBP transcript always produced two discrete amplicons. Sequencing of the two MBP amplicons confirmed that they originate from two splice variants of the MBP transcript: Genbank NM_001025291.1 (variant 1) and NM_001025292.1 (variant 2). Taken together, these results indicate that dMSC and dASC co-
cultured with DRG neurons upregulate the genes associated with myelin production that is characteristic of SC in peripheral nerves.

3.3.1 Protein expression
Western blotting using P0-, PMP22- and MBP- specific antibodies showed that all co-cultures expressed myelin protein P0 (28 kDa), PMP-22 (22 kDa) and MBP (22kDa) (Figure 2, paper III) consistent with the results of the RT-PCR. The addition of ascorbate to the culture medium did not influence myelin expression at either the transcript or protein level (data not shown). Isolated DRG neuron cultures alone did not express significant levels of myelin proteins (Figure 2, paper III), which taken together with the RT-PCR results, confirmed that dissociated neuronal cells did not display any contamination with satellite cells, which could have given rise to spurious results. Isolated SC, dMSC and dASC expressed the proteins P0, PMP22 and MBP (Figure. 2, paper III). MBP was present as single band in Western blots of co-cultures, isolated SC and stems cells, possibly indicating the translation of only one splice variant of the transcript.

3.3.2 Morphological Investigations
Immunostaining for neuronal and glial markers showed the close proximity between neurons and differentiated stems cells in co-culture (Figure 3B, paper III). The results showed that after two weeks in co-culture there was close alignment of S100 positive dASC along neurites extending from neuronal cell bodies. Consistently, immunostaining for P0 of dMSC and dASC showed positive-staining cells closely associated with the neurites (Figure 3A, paper III). Such alignment was also corroborated by the SEM results, which showed dMSC closely aligned with DRG neurites (Figure 4, paper III). The SEM results also confirmed that the size and shape of the differentiated stem cells was consistent with those of SC. The alignment of dASC along the neurites was similar that seen with DRG-SC co-cultures (data not shown), thus reflecting the similarity in spatial relationship between the two cell types with the neurites. These results are further evidence of the functional similarity between SC and differentiated stem cells.

3.4 Qualitative RT-PCR analysis of Notch expression (Paper IV)
RT-PCR analysis showed that untreated proliferating ASC expressed notch-1, notch-2, jagged-1, hes-1 and hey-1 mRNA, but not delta-1 mRNA (Figure. 1A, paper IV). ASC treated with a protocol to induce differentiation to the SC phenotype maintained expression of notch
receptors and hes-1, but following retinoic acid treatment there was a progressive reduction in jagged-1 and hey-1 levels (Figure. 1A, paper IV). In contrast, the delta-1 ligand was up-regulated at the end of the differentiation process. When compared to differentiated ASC, SC showed relatively high levels of notch-1, jagged-1 and delta-1 mRNAs (Figure. 1A, paper IV). When ASC were treated with the gamma-secretase inhibitor, DAPT, there was a noticeable reduction in the transcript levels of the notch responsive gene, hes-1 (Figure. 1B, paper IV); indicating notch signalling could be functionally inhibited.

3.4.1 Proteins analysis
ASC were differentiated in the absence or presence of DAPT notch inhibitor for a period of 2 weeks and then assessed by immunocytochemistry and Western blot analysis for the expression of glial GFAP and S100 proteins (Figure 2, paper IV). GFAP was expressed at varying intensity throughout the differentiated ASC population and was co-expressed with S100 protein in 48.05±7.29% of the cells (Figure. 2A, paper IV). There was no significant difference in the expression levels when the cells had been treated with DAPT, which was confirmed by Western blot densitometric analysis (Figure. 2B, paper IV). Lysates from ASC differentiated both in the absence or presence of DAPT were probed with an S100 antibody and showed a 15 kDa band of similar intensity: this band was absent from the undifferentiated ASC lysates. A 55 kDa band corresponding to GFAP was observed in both sets of differentiated cell lysates, but absent from undifferentiated samples (Figure 2B, paper IV). SC lysates showed an additional lower molecular weight band, which is likely to represent a proteolytic GFAP fragment or alternate transcript. When growth factors used for the differentiation process were removed after the 2-week treatment, expression of SC markers was maintained suggesting a stable SC phenotype (data not shown).

3.4.2 Immunostaining results
We have shown that differentiated ASC enhanced neurite outgrowth in a co-culture model with the NG108-15 motor neuron-like cell line, thus we tested the ability of ASC differentiated in the presence of DAPT to perform this function (Figure. 3, paper IV). Control cultures of NG108-15 neurons extended 0.38μm±0.04μm neurites/cell with a mean length of 41.00μm±7.60μm. These parameters were significantly (P < 0.05) enhanced to 1.64±0.03μm neurite/cell with a length of 147.72μm±9.44 μm in the presence of differentiated ASC.
In the presence of DAPT treated ASC there was a small, but insignificant, decrease to 1.29µm ±0.15µm neurite/cell and length 133.48µm ±1.26µm, suggesting notch signalling does not mediate the ability of differentiated ASC to promote neurite outgrowth.

### 3.4.3 Myelin protein transcript after NOTCH treatment

Co-cultures of dASC in contact with DRG neurons were used to investigate whether notch signalling plays a role in the ability of differentiated ASC to produce myelin proteins. The DRG extended many neurites which made contact with the differentiated ASC (Figure 4A, paper IV). Analysis of mRNA by RT-PCR showed that co-culture of dASC with DRG neurons up-regulated expression of P0 myelin protein which was unaffected by the inhibition of notch signalling (Figure 4B, paper IV). Differentiated ASC expressed PMP-22 in the absence of DRG neurons and this was moderately up-regulated by co-culture (Figure 4B, paper IV). DAPT had no effect on the PMP-22 expression levels in the co-cultures, but appeared to up-regulate expression in DRG neurons alone, possibly by influencing the interaction with contaminating satellite cells. Nevertheless, these results indicate that myelin protein expression is likely to arise from the differentiated ASC given the absence of signal in the single DRG neuron cultures.

### 3.5 Quantification of the percentage of Stro-1 positive cells (Paper V)

To assess the proportion of undifferentiated stem cells in the cultures, cell counts were performed to determine the proportion of Stro-1 positive cells as identified by immunocytochemistry staining of uASC and uMSC (Table 1, paper V). The percentage of stem cells identified by this marker cells is higher in the cultures from neonatal and young rats compare to those of the old group; similar results are obtained with both uASC and uMSC. The number of Stro-1 positive cells decreased with increasing age of the animal.

### 3.5.1 Proliferation Assay

The proliferation assay of SC, uMSC, dMSC, uASC and dASC from animals of different ages was carried out for 10 days in culture for each different passages and the proliferation assay was measured at day 7.

SC: For neonatal SC, increasing passage number does not influence the proliferation rate of the cells (Figure 1A, paper V). The proliferation of the young SC was statistically less between P1 vs P10 (p<0.05), P15 (p<0.01), P20 (p<0.001) and also between P5 vs P20
Overall, the results indicated a tendency of decreasing proliferation rate with increasing passage number (Figure 1A, paper V). In SC from old rats, the proliferation rate was decreased with an increasing number of passages; there was a statistically significant difference when comparing P1 vs P20 (p<0.05).

ASC: The proliferation assays of neonatal, young and old uASC were similar at different passages within each group, and there were no statistically significant differences between the different passages or age groups (Figure 1B, paper V). Similarly, the results of the proliferation rate of neonatal, young and old dASC were comparable between age groups. Although there was an increasing proliferation rate with increasing passage number in each age group, there were no statistically significant differences between the neonatal and young dASC (Figure 1C). In contrast, the old dASC showed a significant (p<0.05) increase in the proliferation rate when P1 and P20 are compared (Figure 1C, paper V).

MSC: The proliferation rate of the uMSC from neonatal, young and old rats was fairly constant within each age group up to P15 (Figure 1D); after this point, neonatal uMSC showed an increased proliferation rate compared to the young uMSC, and there were statistically significant differences between the two age groups at P20 (p<0.01) (Figure 1D). The proliferation rate of the old uMSC showed a tendency to decrease with increasing number of passages, and there was a statistical significance when P1 vs P20 (p<0.05) were compared. The proliferation rate of dMSC from neonatal, young and old rats showed a similar pattern and there were no statistically significant differences between the proliferation rates with number of passages or between age groups (Figure 1E, paper V).

3.5.2 Notch-2 receptor gene expression
Qualitative RT-PCR analysis showed that undifferentiated and differentiated ASC and MSC from animals of all ages expressed Notch-2 mRNA at similar level (Figure 2, paper V). Also, all of these cells appear to express Notch-2 receptor gene at a similar level to that of SC from animals of comparable age.

3.5.3 Expression of p38 protein
Western blotting of p38 showed that the protein was present in all the groups of cells analysed and its expression was increased with the aging of rats (Figure 3A and 3C, paper V). However, there was no significant different in p38 protein levels between the neonatal, young
and old SC (Figure 4A, paper V). In the uASC group the expression of p38 increased with increasing animal age and there was a statistically significant difference (p<0.01) in p38 expression when comparing neonatal and old uASC (Figure 4B, paper V). Interestingly, this increase was also seen in the dASC cells, with a statistically significant difference (p<0.05) between the neonatal dASC and old dASC (Figure 4, paper V).

These results would show a similar pattern of p38 expression for ASC from different age groups whether undifferentiated or differentiated. In contrast, the undifferentiated MSC from neonatal, young and old rats showed a similar expression level of p38 (Figure 4D, paper V) with no statistically significant difference between groups. Similarly, there were no detectable changes in p38 expression in dMSC from neonatal, young and old animals (Figure 4E, paper V).

### 3.5.4 Expression of p53 protein

Western blot analysis of p53 protein in SC, uASC, dASC uMSC and dMSC isolated from neonatal, young and old animals showed no detectable differences in the protein level (Figure. 5A, paper V). However, analysis of the blots showed there was a progressive increase of p53 levels with increase in animal age (Figure 5, paper V). For the majority of cell types this increase was not statistically significant, except for dASC where there were significant differences in p53 levels in cells of neonatal vs young (p<0.01), young vs old (p<0.01) and neonatal vs old (p<0.001) rats.

### 3.5.5 DRG neuron co-cultures

Immunocytochemical staining for βIII- tubulin was used to show the extent of neurite outgrowth from dissociated DRG neurons co-cultured with SC or differentiated ASC and MSC from rats of different ages. In neonatal cell-DRG co-culture the DRG neurite outgrowth was similar for the three types of cells although the length of neurites obtained in co-culture with dMSC was slightly lower compared to the values obtained with the other two cell types (Figure 6A, paper V). In the co-cultures of cells from young animals (Figure 6B, paper V) there was a significant increase in neurite length (p<0.001) when dMSC were co-cultured with the DRG compared to co-culture with SC and dASC. There was no significant difference in neurite lengths between co-cultures with SC and dASC. The DRG were also responsive to co-culture with old SC, dASC and dMSC. The neurite length quantification showed a significantly longer neurite length when DRG were stimulated by old dASC (p<0.001) and dMSC (p<0.001) compared to old SC.
There was no significant difference in the length of neurites when co-culture of DRG neurons with old dASC and dMSC were compared (Figure 6C, paper V).

3.5.6 MitoTracker® staining of mitochondria

Mitotracker® Red staining was carried out to determine the pattern of distribution of mitochondria, which indicate their fission or fusion state. The mitochondrial staining was carried out for SC, uMSC, dMSC, uASC and dASC from neonatal and old rats. In neonatal SC (Figure 7A, paper V), uASC (Figure 8A) and uMSC (Figure 9A, paper V) mitochondria were distributed throughout the cytoplasm and showed a linear distribution [fusion] typical of healthy cells. In contrast, mitochondria in the old SC (Figure 7C, paper V), uASC (Figure 8E, paper V) and uMSC (Figure 9E, paper V) were localised around the nucleus and appeared fragmented and clumped together, like those found in the pre-apoptotic stage cells [fission]. However, in the cell periphery there was still some linear distribution of the mitochondria. Neonatal dASC (Figure 8C, paper V) and dMSC (Figure 9C, paper V) cells displayed a strong and evenly distributed MitoTracker® fluorescence staining comparable to that found in neonatal SC (Figure 7A, paper V). In old dASC (Figure 8G), paper V and dMSC (Figure 9G, paper V) cells, most cells showed only weakly stained mitochondria, mainly fragmented in the cytoplasm or localized around the nucleus as seen in old SC (Figure 7, paper VC), which indicates a possible pro-apoptotic stage during aging.

3.5.7 Electron microscopy - cell morphology

SC of young and old rats appeared very different in the ultrastructural analysis. Young SC displayed a rounded and euchromatic nucleus, usually with no nucleolus (Fig 9A, paper V), with a cytoplasm rich in mitochondria and small isolated elements of the rough endoplasmic reticulum (Fig 9B, paper V). In contrast, the old cells had a lobular and heterochromatic nucleus (Fig 9C, paper V) surrounded by a dense and less organized cytoplasm with few organelles, many vacuoles (Fig 9D, paper V) and a membrane with a greater number of pseudopodia (Fig 9C, paper V) compared to the membrane structure of the young SC. Stem cells isolated from adipose tissue of young rats exhibited an eccentric and lobed nucleus, with one or more nucleoli (Fig 10A, paper V). The nucleus showed mainly euchromatic with a heterochromatin layer distributed along the inner side of nuclear membrane. In these cells, the cytoplasm looked abundantly rich in organelles, mitochondria and endoplasmic reticulum (Fig 10B, paper V). The plasma membrane of young cells had many pseudopodia, which is indicative of a great capacity for adhesion and migration (Fig 10A, paper V): compare this to
the old ASC (Fig 10C, D, paper V) where the pseudopodia were less evident and the membrane more pronounced and regular. In the old ASC the nucleus is irregular (Fig 10C, paper V) and the cytoplasm showed abundant vacuoles containing fat, a typical characteristic of senescent cells (Fig 10D, paper V). Stem cells from bone marrow of young and old rats have similar characteristics to the ASC cells. Young MSC displayed a large pale lobular nucleus with a large amount of euchromatin and noticeably nucleoli (Fig 11A, paper V). Dilated cisterns of rough endoplasmic reticulum were present in cytoplasm with a well organized mitochondrial structure (Fig 11B, paper V). The old MSC displayed an irregular nucleus with a disorganised cytoplasm and mitochondria with thin cristae and early signs of ultrastructural disruption (Fig 11D, paper V).
4.0 Discussion

In the study and application of tissue engineering it is fundamental to find a good correlation between the keys elements that influence the regeneration of the nerve that is the conduit, the transplanted cells, the matrix in which the cells are re-suspended and the growth factors that are involved in the regeneration process.

By definition a “suitable conduit” implies characteristics such as biodegradability must be practical use in clinical application and be non-immunogenic for the patient. This and previous study have shown that the poly-hydroxy-butyrate (PHB) seems to have most of the qualities described above when used in an animal model (Mohanna, Terenghi et al. 2005) and in a recent clinical trial (Aberg, Ljungberg et al. 2009).

However, the main goal is to speed up the regeneration of the nerve with a full recovery of functions so that target organs can be re-innervated before atrophy sets in. A possible solution to obtain this result is the addition of cells and growth factors in the graft to augment the peripheral nerve regeneration. Schwann cells (SC) are the ideal cell type to transplant into the site of injuries (Mosahebi, Simon et al. 2001). Due to the many difficulties of sourcing and culturing SC, studies have focused in finding an ideal cell substitute, and bone marrow and adipose derived stem cells appear to be promising alternative candidates. They are reasonably easy to harvest and culture in vitro, and because of their plasticity, they can differentiate along multiple lineages when exposed to growth factors. In particular, MSC and ASC will differentiate into cells expressing neuronal markers when exposed to a glia growth factor (GGF). Evidence of the differentiation of MSC into SC-like cells has been shown (Tohill and Terenghi 2004) and studies of their morphological, phenotypical and molecular characterization have been published more recent (Caddick, Kingham et al. 2006; Kingham, Kalbermatten et al. 2007; Mahay, Terenghi et al. 2008).

One of the main issues in cell transplantion is the need to track and evaluate the survival and integration of the cells in vivo. The development of new techniques, such as fluorescent green proteins, has considerably improved the study of bio-engineering cell systems (Tohill, Mann et al. 2004). The study in Paper I has shown that it is possible to transfect efficiently and stably primary SC cultures with green fluorence protein (GFP) using a retroviral vector, and that established cultures of transduced cells expressing GFP display a proliferation and viability profile similar to non-transduced cells. Previous work in our group used the MMLV vector to transduce SC with the lacZ gene (Mosahebi, Simon et al. 2001).
The advantage of using the GFP is the capability of identifying the cells and following their growth without the need of fixation as required when using the lacZ technique. During multiple passages of in vitro culturing, the level of expression of GFP did not decrease indicating a stable expression of the gene in SC genome and reinforcing the advantage of using an exogenous fluorescence gene for cell labeling. We have shown that GFP can be visualized in combination with other fluorochrome-labelled antigens, and to be suitable for cell identification using fluorescence immunohistochemical staining. GFP protein is also unaffected by acids, proteolytic enzyme and temperature up to 65°C (Bokman and Ward 1981). However, because the protein is soluble, it requires formaldehyde fixation prior to cryostat sectioning. At present many companies produce a range of different fluorescence proteins to allow simultaneous multicolor tracking of labelled cells. Mutants of GFP (EGFP) are now available with an emission corresponding to blue (BFP), cyan (CYP) and yellow (YFP) (Zimmer 2002). This will give scope for further expansion of investigative tools in the future.

The study in Paper II has shown that bone marrow stem cells express SC morphological markers after in vitro differentiation using GGF. This is a well known Schwann cell mitogen and it stimulates differentiation towards a glial cell lineage. Neuronal cells are the natural source of GGF and it is possible that the secretion from the regenerating neurons may have stimulated the in vivo differentiation of some uMSC.

The MSC development pathway is influenced by both intrinsic and extrinsic factors combined together, which that control the molecular and cellular pattern of expression in the different tissues. In vivo, the process of differentiation from stem cell to the final phenotype pattern is composed of different stages dependent upon the stimuli received from different sources. The signals can originate from the extracellular matrix and from the local signalling by surrounding cells (paracrine regulation), but the stem cells are also influenced by signals released by the stem cells (autocrine regulation). The microenvironment is thought to control stem cell behaviour based upon correlations of events such as cell-cell interactions, secreted factors and the extracellular matrix (Jan and Jan 1998). Based on the combinations of intrinsic and extrinsic stimuli, MSC develop their final morphology in the specific tissue in which they are implanted. Some of the events have been studied by other groups (Marion and Mao 2006), but most of them remain unclear and unknown. In this study we showed that MSC, with appropriate in vitro stimulation, can exhibit differentiation into SC and support nerve regeneration. It could be argued that it is not clear if the MSC were integrated into the tissue because of the GGF stimulation in vitro or because the differentiation is due of intrinsic and
extrinsic signals received from the tissue in which they had been transplanted. We can speculate that these tissue-derived factors may be responsible for some of the undifferentiated stem cells transplanted in PHB conduits and used as a control, which showed positive expression for the S100 marker *in vivo*. Based on these theories, cell culture conditions become fundamental to understand not only the progression of events that occurred *in vitro*, but also provide a better control of the cells *in vivo*. For this reason it is essential to carefully track the cells using a GFP-transfection technique that was established in previous work (Tohill, Mann et al. 2004). This method allows determination of the position of the cells in the host. Furthermore, dMSC transplanted *in vivo* positively influence axonal and SC cell regeneration (Tohill and Terenghi 2004).

This study shows that dMSC effect on improving regeneration is consistent when compared to Schwann cell transplantation. The co-localization of GFP-dMSC and the expression of PGP9.5 markers displayed an integration of regenerating axons and transplanted marrow stem cells. Moreover, it also showed a homogeneous distribution of the dMSC throughout the conduit from the proximal to the distal stump.

Paper III presents good evidence that dMSC and dASC are morphologically and functionally similar to SC. In our co-culture model, we recreated the type of environment that stimulates SC to produce myelin whilst they are in contact with neuronal cells. Using this model we were able to demonstrate the expression of the major myelin proteins, P0, MBP and PMP-22 by dMSC and dASC. When adult differentiated stem cells are co-cultured with adult DRG neurons they are able to express transcripts and proteins of peripheral nerve myelin. Although electron microscopy showed the alignment of differentiated stem cells along the neurites, it was not possible to demonstrate the formation of myelin around the neurites. This is likely due to the short time that these co-cultures can be kept, which may allow expression of myelin protein, but it is not sufficient for the glial cell to fully envelop the neurites and develop myelin sheets. Myelin formation was demonstrated in the PC12 neuronal cell line and dMSC co-cultures at two weeks (Keilhoff, Stang et al. 2006), but myelin formation was highly dependent on culture conditions. Furthermore, the use of neuronal cells derived from a tumour and the use of dissimilar differentiation and culture medium makes the comparison with our results difficult. Also, expression of different myelin proteins was not examined, nor the use of dASC as shown in our experiments.

Myelin is a complex structure produced by SC in the PNS and its development is strictly correlated with the interactions of SC and axons. In a SC-DRG co-culture model, it was
shown that neurotrophins enhance myelin formation (Martini 1998) with NGF and BDNF being positive modulators of myelination during embryogenesis. Similarly, in our study the addition NGF and BDNF was essential to stimulate neurite extension and myelin protein expression and this was confirmed by experiments where omission of either or both of these neurotrophins resulted in a negative outcome (results not shown). Recently, it was speculated that embryonic DRG neurons could induce phenotypic transdifferentiation of MSC into SC-like morphology and stimulate expression of antigens such as S100 (Yang, Lou et al. 2008), but the co-culture of these cell types was not sufficient to elicit myelin expression in vitro.

In vivo, the myelination process is based on the strict association between myelinating SC and axons involving different reactions, including formation of basal lamina. Previous in vitro studies showed that the ability of SC to assemble basal lamina and proceed to myelination is dependent upon culture conditions, including the requirement for ascorbate (Obremski and Bunge 1995). Varying ascorbic acid concentration affected MSC differentiation, proliferation, cytotoxicity and ECM production, and ascorbate is a potent stimulator of MSC proliferation, without reducing their differentiation capacity (Choi, Yoo et al. 2008).

P0 is the most abundant protein expressed by myelinating SC in the PNS, but a low basal level of P0 expression in SC and neuronal crest cells is seen in early embryonic development (Jessen and Mirsky 1999). The development of myelin implies an active interaction between neuronal and glial cells. Consistently, our RT-PCR data show that dMSC and dASC do not express P0 transcripts when cultured in the absence of neuronal contact, while immunocytochemical staining of the dMSC-DRG co-cultures provided evidence that contact is a fundamental requirement to stimulate expression of P0 protein. The up-regulation of P0 by SC during myelination appears to involve as yet unidentified signals from the axon, interactions with the basal lamina and elevated levels of intracellular cAMP (Fernandez, Pena et al. 2002). In our study, we have shown that, PMP-22 is also expressed by dMSC and dASC. This supports the hypothesis that expression of P0 and PMP-22 is linked during the formation of the PNS myelin. The PMP-22 protein appears to play a role both in formation and maintenance of myelin (D'Urso, Ehrhardt et al. 1999).

Myelin basic protein (MBP) is reported to be essential for myelin compaction and formation of the myelin dense line (Yin, Kemp et al. 2001), and in CNS there is a strict correlation between the expression level of MBP and myelin thickness (Shine, Readhead et al. 1992). However, MBP absence does not prevent the formation of compact myelin in PNS as this absence seems to be compensated by another myelin protein, possibly P0. It has been observed that P0 and MBP can play interchangeable roles during myelin formation in the
dense line (Martini and Schachner 1997). The results of our study showed expression of two isoforms of the MBP transcript whereas Western blot showed only one protein to be present. Following sequencing of the two transcripts, we concluded that only one isoform is expressed. The disparity between the number of MBP isoform transcripts and the number of detectable MBP proteins remains to be clarified, but the strong expression of P0 protein might be indicative of a compensatory effect for the absence of one of the two isoforms of MBP.

These results confirm that adult dMSC and dASC can transcribe and translate myelin protein in a similar manner to SC. These results clearly indicate a functional similarity of differentiated adult stem cells to mature Schwann cells.

In paper IV we have been the first to document the molecular expression of a range of notch signalling molecules in adipose derived stem cells and show that there is dynamic expression during differentiation to a SC phenotype. Expression of jagged-1 was markedly down-regulated during the differentiation process and a study of neuron differentiation of bone marrow MSC showed a similar phenomenon (Xing, Bai et al. 2007). In vivo studies indicate that jagged-notch signalling is required to maintain proliferation of neural progenitors (Yeo and Chitnis 2007) suggesting the down-regulation of jagged could act as a switch towards neuro-glial differentiation. However, in contrast to our SC-like cells, primary SC showed relatively high levels of jagged-1 expression. Though there are no reports in the literature of jagged function in SC it is interesting to note that jagged was first cloned from a rat SC library (Lindsell, Shawber et al. 1995). Hes-1 was expressed in both undifferentiated and differentiated ASC and the expression levels did not appear to change at key stages of the differentiation protocol. DAPT a gamma-secretase inhibitor which prevents processing of notch receptor completely abolished hes-1 transcript levels in control proliferating cells and markedly down-regulated expression in differentiated cells. This strongly suggests that notch signalling is active in ASC but it is also possible that hes-1 expression in SC differentiated cells could in part be regulated by other notch independent signalling pathways described in other cell types (Curry, Reed et al. 2006). DAPT has also been shown to inhibit the self-renewal/proliferative capacity of bone marrow mesenchymal stem cells and significantly reduce the ability of these cells to undergo chondrogenic differentiation (Vujovic, Henderson et al. 2007). Given the evidence that ASC express notch signalling molecules we examined the effect of DAPT on the differentiation expression of glial cell markers. DAPT treatment of neural stem cells has been shown to reduce the ability of the cells to differentiate into GFAP positive cells (Cai, Lin et al. 2008) and another recent study indicates that notch activation is
critical for glial differentiation of mesencephalic neural crest cells (Fiuza and Arias 2007). In contrast, we found that inhibiting notch signalling had no effect on the expression levels of glial proteins in differentiated ASC-like cells. Consistent with this we found that DAPT treated SC-like cells enhanced neurite outgrowth of a neuronal cell line to a similar extent of cells differentiated in the absence of inhibitor. These results argue strongly against the notion that differentiation of ASC into SC-like cells represents a regulated process analogous to the pathways of intrinsic neuro-glial cell progenitor differentiation. ASC have recently been shown to myelinate PC12 cells in vitro (Xu, Liu et al. 2008) and it was shown that human bone marrow stem cells differentiation to O4 antigen positive myelinating glia cells could be enhanced by signalling through an F3 neural cell adhesion molecule/notch interaction (Lu, Chen et al. 2008). Furthermore, using transgenic animals it was found that notch inactivation leads to premature myelination (Woodhoo, Alonso et al. 2009). Thus it can be concluded that in primary SC notch plays a critical role in myelination. In contrast, our studies suggest that inhibition of notch had no effect on myelination evoked by differentiated ASC. Thus the signalling pathways mediating the neurotrophic activity and myelination capacity of ASC remain to be determined.

Adult stem cells have been shown to be capable of differentiation into various lineages and the effect of donor age and the in vitro handling require to be investigated in order to avoid pitfalls in the development of cell based therapies. In study V, SC, MSC and ASC isolated from sciatic nerve, bone marrow and adipose tissue, respectively, from rats of different ages ((neonatal, young (four months) or old (twenty months)) were compared in terms of their in vitro growth kinetics, expression of senescence protein and morphologically structure to investigate if the aging of the cells or number of passages in culture might influence their functionality. Quantification of the stem cell surface marker Stro-1 confirmed that for both MSC and ASC the percentage of Stro-1 positive cells was similar for neonates and young adults, but with a decrease in Stro-1 positive cells from old animals. These data would indicate that the number of stem cells harvested from bone marrow or fat decreases with the age of the host. A proliferation assay was used to compare the growth characteristics of the same cells for up to twenty passages. In neonatal SC the number of passages does not appear to influence the growth rate of the cells although in SC from both young and old animals the cell growth tends to decline with the increasing passage number. The major differences appeared in the young and old animal cell group, where the number of passages affected the SC proliferation. In particular, in old SC a difference between the initial and the final
passages (P1 and P20) is evident and indicate a decreased in cell proliferation over time. Generally, in the MSC and ASC groups the slope of the growth curve is similar among the different age groups. Neonatal MSC and ASC are not affected by the increase of number of passages and their growth is fairly constant for both undifferentiated and differentiated cells. Similarly, the proliferation remains constant in the groups of young MSC and ASC.

The uMSC and uASC from old rats showed a similar decrease of proliferation rate with increase in passage number (P20). Although the number of passages is not sufficient to evaluate the decreasing aging process, the uMSC and uASC from old rats showed initial signs of senescence age-related that is consistent with the morphological change found by electron microscopy. However, following differentiation, this decline in proliferation rate appears to be reversed as there were no major differences in the growth curves between the three age groups; however, there was a significant increase in proliferation rate with number of passages observed for dASC. A possibly explanation for this observation could be the influence of the growth factors used for the in vitro differentiation process. As demonstrated elsewhere, restoration of mitogenic and growth signaling may rejuvenate cells and instead of entering in a senescence cycle the cells respond to a growth factor induced ‘reprogramming’ that leads them to maintain proliferation despite the high passage number (Mayack, Shadrach et al. 2010).

The change in proliferation rate after differentiation seems to be in contrast with the results of recent studies, which demonstrated that MSC harvested from donor animals of different ages and cultured for different numbers of passages showed a decrease in their chondrogenic and osteogenic potential with increasing passage number, whereas their adipogenic potential decreased with donor age (Kretlow, Jin et al. 2008). However, others have shown that there are no age related differences in rat MSC differentiation or changes in proliferation, attachment and senescence (Baron, Metz et al. 2000; Bellows, Pei et al. 2003; Tokalov, Gruner et al. 2007; Coulthard, White et al. 2009). It has been established that the p38 MAP kinase pathway is activated in response to physical stress signals, such as osmotic shock, heat, UV light, and in response to pro-inflammatory cytokines, such as TNF-α, IL-1 and growth factors like bFGF and PDGF (Baron, Metz et al. 2000). In this study, the expression of p38 protein was investigated in the different cell types isolated from animals from the three different age groups. Quantification of p38 protein expression showed there to be a slight upward trend in expression level with increasing age of the donor animal. While in the groups of SC and MSC (undifferentiated and differentiated) the expression of p38 increased
but it was not statistically significant, in the uASC and the dASC groups the expression of p38 was more evident and it was statistically significant in cells from old rats. This result could be a further indication that the cellular senescence may be linked to the age of the donor. Interestingly, in the dASC there are significant differences in p38 expression levels when neonatal rat cells are compared to young and old rat cells, but the difference is not significant when comparing the young to the old group. Although in the group of uASC the difference could be attribute mainly to the age of donor, in the dASC it could be hypothesized an involvement of the growth factors, such as the FGF2 and PDGF used for the differentiation and maintenance of the stem cells, could affect the expression of the p38 and consequently cell proliferation (Baron, Metz et al. 2000). Indeed, p38 has been implicated in cell cycle arrest, DNA repair and programmed cell death (Cohen, Ciocca et al. 1997; Robinson and Cobb 1997; Morooka and Nishida 1998); p38 activation has also been associated with the cellular response to stress rather than proliferation (Mohammadi, Dikic et al. 1996; Cohen, Ciocca et al. 1997; Robinson and Cobb 1997; Morooka and Nishida 1998).

The protein p53 regulates cell cycle progression and apoptosis, but it can also directly modulate the transcription of genes that are specifically required for neuronal differentiation (Tedeschi and Di Giovanni 2009; Molchadsky, Rivlin et al. 2010). The function of p53 in development and its importance in the control of differentiation processes have been previously established with some discrepancies. Some studies suggest that p53 facilitates cell differentiation whereas in others it seems to be suppressive (Almog and Rotter 1997; Choi and Donehower 1999). Our study showed that the expression of p53 protein in all cell types examined (SC, uMSC, dMSC, uASC and dASC) from the three different age donors did not differ significantly. Interestingly, the level of the expression of p53 protein appears to be slightly increased in both differentiated ASC and MSC compared to the undifferentiated cells. Mutations in p53 have been implicated in age-related mesenchymal stem cell transformation and in the self-renewal and differentiation of neural stem cells (Li, Fan et al. 2007; Armesilla-Diaz, Bragado et al. 2009). The activity of p53 protein was initially thought to be limited to the nucleus, although recently it became apparent that p53 also regulates mitochondrial response to stress stimuli (Erster, Mihara et al. 2004; Compton, Kim et al. 2011). However, from the normalized results we obtained in our study, p53 does not appear to significantly influence the biology of Schwann or stem cells.

In this work, DRG-neuron co-cultures were used as a bioassay to assess neurite outgrowth as indicative of growth factor secretion following differentiation of ASC and MSC into SC-like
Many studies have shown that untreated DRG neurons fail to extend neurites during the first day in culture and neurite extension is influenced by trophic factor (Cafferty, Gardiner et al. 2001). Previous studies have shown that the DRG neurons are able to extend neurites in the absence of exogenous NGF, an effect due to soluble factors release by the cells (Caddick, Kingham et al. 2006). Moreover, it was demonstrated that the secretion of BDNF and NGF is not intrinsic to stem cells, but due to their differentiation to the glial cell lineage (Mahay, Terenghi et al. 2008). Consistently, the indirect co-culture used in system this study has shown that growth factors secreted by SC and differentiated MSC and ASC elicited neurite outgrowth independently of the age of the donor animal. In the neonatal group there were no significant differences in the DRG neurite lengths after co-culture with neonatal SC, dASC and dMSC. Similar results were obtained with co-culture of SC and dASC from young animals, but in contrast, dMSC appeared to stimulate more neurites growth. There is a statistically significant increase in the length of neurites in the co-cultures of DRG neurons with dASC or dMSC derived from old rats compared to those with SC from animals of the same age. It is well known that nerve regeneration is dependent on a secretion of growth factors from glial cell and these results would be indicative of the possibility of using stem cells from older individual to be used for cell therapy of nerve repair (Terenghi 1999). The discrepancy between the results of DRG neuron co-cultures with neonatal cells and old cells is notable and unexpected. However, increased expression of p53 may be indicative of an increase in cellular senescence and therefore to a reduced functionality of the cells, it has been shown that cellular senescence as defined by the loss of proliferative potential is also linked to cellular over-activation and correlates with cellular hypertrophy (Demidenko, Zubova et al. 2009). In the case of differentiated stem cells, this over activation might correlate to increase growth factors secretion and consequently to stimulation of neurite outgrowth. Degenerating events such as oxidative stress, exposure to UV light and aging culminate in the loss of cells functionality. Mitochondrial dysfunction in aged stem cells may provide indicative information for their applications in tissue engineering therapies. Considering that mitochondria are central in the process of apoptosis, this study focused on investigating both the location and gross morphology of the mitochondria present in the neonatal, young and old animal derived ASC and MSC. Mitochondria were recognised for their dynamic morphology, which changes to meet the metabolic needs of the cell, an event that is regulated by two main processes, mitochondrial fission and fusion (Lee, Jeong et al. 2004). In mammalian cells, the mitochondrial population aligns in strategic subcellular positions to form long interconnected networks by mitochondrial fusion. Conversely, in
immature or apoptotic cells, the mitochondria increase in numbers and appear as short motile, unconnected units defined as mitochondria fission. A firm link between mitochondrial dynamics and cell functions has yet to be established. Fragmentation of mitochondria during cell death has been shown to play a key role in cell death progression, including the release of the mitochondrial apoptotic proteins (Cheung, McBride et al. 2007). Ultrastructural changes in mitochondria, such as cristae remodelling, is also involved in cell death initiation and initial sign of senescence (Ulivieri 2010). In our study mitochondrial changes in cells from animals of different ages were evident as shown by fluorescence staining and by electron microscopical analysis. Mitochondria of cells from old rats showed distinct differences in morphology, such as a reduction of fusion and increased fission, compared to those from neonatal animals. In SC, ASC and MSC from old animals most mitochondria were clustered around the nucleus compared to the neonatal cells. The aggregation of mitochondria in the perinuclear region of postmitotic cells may be for a variety of reasons. Mitochondria in mammalian cells are moved mainly along microtubules (De Vos, Severin et al. 2000), and disruption of fission protein Drp1 resulted in perinuclear clustering of mitochondria (De Vos, Severin et al. 2000; Varadi, Johnson-Cadwell et al. 2004). Perinuclear clustering of mitochondria has also been observed in apoptosis as well as in necrosis (Thomas, Zhang et al. 2000). Furthermore, electron microscopical studies of young and old undifferentiated and differentiated ASC and MSC, showed mitochondrial degeneration and loss of the cristae, indicating a reduced amount of available inner mitochondrial membrane in the old animal cells. A diminished expression of respiratory chain complexes could be a consequence of these structural changes, resulting in a reduced membrane potential (Jendrach, Pohl et al. 2005). Taken together, the reduced mitochondrial fusion and their perinuclear clustering might indicate decreased energy production in ageing cells. Overall the results of this study show that aged MSC and ASC differentiated into SC-like retain the potential to support axon regeneration. Although the aged animal cells characterized in this study expressed markers, such as p38 and p53, typical of senescence and the intensity of the staining of the mitochondria was decreased maybe due to a reduction in mitochondria activity, these features do not seem to affect the cell proliferation rate or their regenerative function. Other studies have shown that age does not affect the ability of MSC and ASC to support the regeneration process following injury (Morrison and Costanzo 1995; Luo, Geng et al. 2010). The increased understanding of the biological mechanisms of MSC and ASC derived from donors of different ages will expand their functional applications in tissue engineering and maximize their therapeutic use.
5.0 Conclusions

In the study and application of tissue engineering to nerve repair it is fundamental to pinpoint the key elements that influence the regeneration of nerve fibres: primarily, these are the type of cells that naturally promote neurite sprouting and the appropriate permissive microenvironmental conditions for regeneration of the damaged tissue. Enriching a site of peripheral nerve damage with cultured Schwann cells (SC) has been shown to improve nerve regeneration, but this approach is limited by the difficulty in sourcing clinically useful numbers of autologous cells. Clearly, an alternative easily sourced and expanded substitute cell type is needed and adult bone marrow mesenchymal stem cells (BM-MSC) and adipose derived stem cells (ASC) have the potential to fulfil that role. These stem cells have the capacity for self-renewal and multi-lineage differentiation and play an important role in regenerative medicine. However, the loss of stem cell functionality is accompanied by a range of age-related biochemical changes. Moreover, it is possible that donor age could influence the properties and efficacy of these cells in tissue repair.

On the basis of the experimental findings presented in this thesis, the following conclusions can be drawn:

- It is possible to efficiently and stably transfect primary SC cultures with green fluorescent protein (GFP) using a retroviral transduction. The proliferation and viability profile of the GFP-expressing cells is similar to that of non-transduced cells. Furthermore, GFP labelling these cells aided in their identification following transplantation into and in vivo nerve repair model system.
- With appropriate in vitro biochemical stimulation, BM-MSC and ASC demonstrate the ability to cross lineage boundaries and undergo glial differentiation.
- BM-MSC can differentiate into SC-like cells morphologically and functionally identical to native SC and have the ability support peripheral nerve regeneration through PHB conduits in vivo.
- BM-MSC and ASC differentiated into SC-like cells are able to express the main proteins fundamental to myelin formation in the PNS: this property is of crucial importance if glial differentiated BM-MSC or ASC are to be used as SC substitutes to enhance peripheral nerve regeneration in vivo.
- Although Notch signalling plays a key role in primary SC function inhibition of this pathway had no effect on ASC differentiation towards a SC-like cell even though the specific signalling pathway involved remain to be determined.
- The morphological, molecular and functional properties of SC, MSC and ASC isolated
from rats of different ages were almost identical. Moreover, despite expressing markers associated with cellular senescence, the MSC and ASC from aged animals retained the ability to differentiate into SC-like cells and support axon regeneration to a level comparable to that of cells from young and neonatal animals.

In summary, this thesis has shown that glial differentiated BM-MSC and ASC are potentially useful in the repair of damaged nerves and the effect of donor age on cell viability and function should not be a constraining factor for their application in the clinical situation.
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“Man’s life consists in the affection which sustains him most, for there he finds his greatest satisfaction.”

St. Thomas Aquinas

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REFERENCE


