SKIN-DERIVED PRECURSORS ARE A SUITABLE ALTERNATIVE TO
PERIPHERAL NERVE AS A SOURCE OF SCHWANN CELLS FOR
TRANSPLANTATION-BASED REPAIR OF THE INJURED RAT SPINAL CORD

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

February 2014

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Abstract

For much of human history the devastating loss of neurological functions that occurs after spinal cord injury (SCI) was thought to be irreversible, so the people afflicted with such injuries were offered no hope of effective medical treatment. Today that has changed, as advances in neurobiology and medicine over the past century have led to the development of treatments aimed specifically at repairing the injured spinal cord. The transplantation of Schwann cells (SCs) has emerged as one promising example of such a treatment, with demonstrated efficacy in multiple animal models of SCI and encouraging preliminary results in clinical trials. Although SCs possess many of the qualities of an ideal cellular therapy, the harvest of autologous SCs from peripheral nerve (N-SCs) causes permanent nerve injury, which could be avoided by generating SCs from an alternative autologous source. One such source is skin-derived precursors (SKPs), which can be isolated from the adult mammalian dermis and differentiated into SCs (SKP-SCs) in vitro.

Herein I examined the efficacy of SKP-SCs as a treatment for SCI in rodent injury models and compared those cells to their nerve-derived counterparts. This work provided the first demonstration of efficacy for SKP-SC therapy after thoracic contusion and showed that, much like N-SCs, SKP-SCs myelinate, promote axonal growth, and enhance functional recovery after SCI. In addition, we found evidence that SKP-SCs may have advantages over N-SCs with respect to their ability to interact favourably with spared astrocyte-rich host tissue and promote axonal growth. Subsequently we directly compared neonatal SKP-SCs and N-SCs and found that those cell types were highly similar in terms of their protein/gene expression profiles, migration and integration into astrocyte-rich domains in vitro and in vivo, and many reparative effects following transplantation into the partially crushed cervical spinal cord. Taken together our
findings suggest that SKP-SCs and N-SCs have similar therapeutic efficacy, and that where differences between those two cell types exist, they consistently favour the SKP-SCs as the more favourable cell type for SCI repair. Thus, our work to-date supports the notion that SKP-SCs are a suitable alternative to N-SCs for transplantation-based central nervous system repair.
Preface


Jeff Biernaskie and I shared co-first authorship on that publication as we contributed equally to the work. The original idea for this study came from Freda Miller and Wolfram Tetzlaff, who also supervised the *in vitro* and *in vivo* aspects of this collaborative study, respectively. I assisted with experimental design and I was chiefly responsible for planning and organizing many aspects of the experiment, including: all surgeries, animal care, and behavioural testing (including 5 measures), and some of the *in vivo* histological assessments. I also assisted with contusion surgeries, conducted or supervised all aspects of animal care and behavioural testing, and cut, immunostained, and/or imaged a substantial portion of the tissue used for histological quantifications and figure images. In addition I conducted the statistical analyses for all of the behavioural data and a portion of the histological data, wrote the sections of the methods and results pertaining to those analyses, made about half of the figures in the paper, and provided feedback on drafts of the manuscript prior to publication.

Jeff Biernaskie conducted all of the cell culture work, delivered and resuspended the cells for transplantation, conducted/supervised a significant portion of the histological analysis, and wrote the bulk of the final paper in collaboration with Wolfram Tetzlaff. Jie Liu performed all of the animal surgeries. Jason Plemel and Casey Shannon assisted me with certain behavioural assessments and with perfusions at the end of the study. Robert Xie provided technical assistance on some of the histology. This work was conducted with the approval of the University of British
Columbia Animal Care Committee. The relevant animal care certificate number is A03-0139
“Anatomical and functional recovery after spinal cord contusion injury”.

A version of Chapter 3 has been prepared for submission to be published\textsuperscript{2}. Sparling JS, Bretzner F, Biernaskie J, Assinck P, Jiang Y, Arisato H, Plunet WT, Borisoff J, Liu J, Miller FD, Tetzlaff W. Neonatal Schwann cells generated from skin-derived precursors or peripheral nerve induce similar levels of functional recovery after transplantation into the partially injured cervical spinal cord of the rat.

The \textit{in vitro} and \textit{in vivo} portions of that work were again supervised by Freda Miller and Wolfram Tetzlaff, respectively. Frederic Bretzner planned, organized and supervised the surgeries, behavioural analyses and histological assessments conducted in experiment 1, and also conducted the electrophysiological assessment in both experiments, quantified all of the rubrospinal tract axon branching data, and wrote an initial paper based on the results of experiment 1. I planned and organized all aspects of experiment 2. In that experiment I assisted with the culture work and resuspended the cells for transplantation, conducted or supervised all aspects of animal care, behavioural testing, and histological assessments, except the rubrospinal tract branching analysis, which was conducted by Frederic Bretzner on images that I took from tissue that I immunostained. I also resampled all of the histological data from experiment 1 (except the RST axon branching data), ran all final data analyses, created all of the figures and wrote the final draft of the manuscript combining the work from both experiments.

Jeff Biernaskie isolated and expanded all of the cells used in this study and resuspended the cells for transplantation in the first study. Peggy Assinck and Yuan Jiang assisted with animal care, behavioural testing and analysis, and/or immunohistochemistry in experiment 2. Hiroki Arisato assisted with animal care and behavioural testing and analysis in experiment 1. Ward
Plunet provided conceptual input and technical assistance on forelimb behavioural measures in experiment 1. Jamie Borisoff provided technical assistance on the electrophysiological measures and Jie Liu conducted animal surgeries in both experiments. This work was conducted with the approval of the University of British Columbia Animal Care Committee. The relevant animal care certificate numbers include: A03-0112 “Regeneration of chronically injured rubrospinal neurons” and A06-1529 “Anatomical and functional recovery after spinal cord contusion injury”.

A version of Chapter 4 is in preparation for publication\(^3\). Sparling JS, Plemel JR, Biernaskie J, Miller FD, Tetzlaff W. Schwann cells generated from neonatal rodent skin-derived precursors are functionally indistinguishable from species- and age-matched nerve-derived Schwann cells.

I designed all of the experiments included in Chapter 4, conducted all of the Schwann cell culture work and \textit{in vitro} assessments, conducted or supervised all of the \textit{in vivo} measures, conducted all of the data analysis, made all of the figures and wrote the manuscript. Jason Plemel kindly provided the astrocytes used for the \textit{in vitro} analysis and provided useful input regarding cell culture issues in general. Jeff Biernaskie trained me to culture Schwann cells from nerve, provided helpful input regarding Schwann cell culture in general, and cultured the Schwann cells used in the \textit{in vivo} work, the latter of which was supervised by Freda Miller. Wolfram Tetzlaff supervised all of the \textit{in vitro} and \textit{in vivo} experiments. This work was conducted with the approval of the University of British Columbia Animal Care Committee. The relevant animal care certificate numbers include: A08-0200 “Myelin inhibition of myelination” and A06-1529 “Anatomical and functional recovery after spinal cord contusion injury”.
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AMCA</td>
<td>7-amino-4-methylcoumarin-3-acetic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP2α</td>
<td>activating enhancer binding protein 2 alpha</td>
</tr>
<tr>
<td>ASC(s)</td>
<td>adipose stem cell(s)</td>
</tr>
<tr>
<td>ASIA</td>
<td>American Spinal Injury Association</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BACE1</td>
<td>beta-site APP-cleaving enzyme 1</td>
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<tr>
<td>BBB</td>
<td>Basso, Beattie, and Bresnahan locomotor scale</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>BCE</td>
<td>before the common/current/Christian era</td>
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<tr>
<td>BDA</td>
<td>biotinylated dextran amine</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>βIII tub</td>
<td>beta III tubulin</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor (aka: FGF-2)</td>
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<tr>
<td>BMP2</td>
<td>bone morphogenetic protein 2</td>
</tr>
<tr>
<td>BMSC(s)</td>
<td>bone marrow stromal cell(s)</td>
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<tr>
<td>Brn2</td>
<td>brain 2</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSCB</td>
<td>blood-spinal cord barrier</td>
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cAMP – cyclic adenosine monophosphate
Caspr – contactin-associated protein
CCL2 – chemokine (C-C motif) ligand 2
CD34 – cluster of differentiation 34
Cdc2 – cell division control protein 2 homologue
Cdc42 – cell division control protein 42
cDNA – complementary deoxyribonucleic acid
CE – common/current/Christian era
CGRP – calcitonin gene-related peptide
ChatABC – chondroitinase ABC
CNPase – 2′, 3′- cyclic nucleotide 3’-phosphodiesterase
CNS – central nervous system
CNTF – ciliary neurotrophic factor
CPG(s) – central pattern generator(s)
CsA – cyclosporine A
CSPG(s) – chondroitin sulfate proteoglycan(s)
CST – corticospinal tract
CT – computerized tomography
CTB – Cholera toxin B
CT dye – CellTracker dye
CXCL10 – chemokine (C-X-C motif) ligand 10
Cy3 – cyanine 3
DβH – dopamin beta-hydroxylase
DCC – deleted in colorectal cancer
DHH – desert hedgehog
DLF – dorsolateral funiculus
DMEM – Dulbecco’s modified Eagle’s medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DPBS – Dulbecco’s phosphate buffered saline
DRG – dorsal root ganglion
EAE – experimental autoimmune encephalomyelitis
ECL – enhanced chemiluminescence
ECM – extracellular matrix
EDTA – ethylenediaminetetraacetic acid
EGF – epidermal growth factor
Egr2 – early growth response gene 2 (aka: Krox-20)
EM – electron microscopy
EMG – electromyography
EPI-NCSC(s) – epidermal neural crest stem cell(s)
ErbB2/3 – erythroblastic leukemia viral oncogene homolog-2/3
ES cell(s) – embryonic stem cell(s)
EYFP – enhanced yellow fluorescent protein
FBS – fetal bovine serum
FDA – Food and Drug Administration
FGF – fibroblast growth factor
Fibro(s) – dermal fibroblast(s)
FITC – fluorescein isothiocyanate
GalC – galactocerebroside
GAP-43 – growth-associated protein-4
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
GDNF – glial-derived neurotrophic factor
GFAP – glial fibrillary acidic protein
GFP – green fluorescent protein
GGF – glial growth factor
GM – gray matter
GRP(s) – glial-restricted precursor(s)
GTPases – guanosine triphosphate hydrolase enzymes
HBSS – Hank’s balanced salt solution
HCl – hydrogen chloride
HDAC(s) – histone deacetylase(s)
hfPS cell(s) – hair follicle pluripotent stem cell(s)
HNK-1 – human natural killer-1
Ho – Hoechst nuclear stain
HSC(s) – hematopoietic stem cell(s)
HSPG(s) – heparin sulfate proteoglycan(s)
Id2/4 – inhibitor of DNA binding 2/4
IGF – insulin-like growth factor
IL – interleukin (e.g., IL-1: interleukin-1)
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>iNPC(s)</td>
<td>induced neural precursor cell(s)</td>
</tr>
<tr>
<td>iPS cell(s)</td>
<td>induced pluripotent stem cell(s)</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity (e.g., GFAP-IR)</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA(s)</td>
<td>micro ribonucleic acid(s)</td>
</tr>
<tr>
<td>MMP2</td>
<td>matrix metalloprotease 2</td>
</tr>
<tr>
<td>mpz</td>
<td>myelin protein zero gene</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MSC(s)</td>
<td>mesenchymal stem cell(s)</td>
</tr>
<tr>
<td>NCad</td>
<td>neural cadherin</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NCSC(s)</td>
<td>neural crest stem cell(s)</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchange protein</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament-200</td>
</tr>
<tr>
<td>Nf1</td>
<td>neurofibromatosis type 1 gene</td>
</tr>
</tbody>
</table>
NFATc4 – nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-4
NF-κB – nuclear factor kappa-light-chain enhancer of activated B cells
NFM – neurofilament M
NG2 – neuron-glia antigen 2
NGF – nerve growth factor
NgR – Nogo receptor
NMDA – N-Methyl-D-aspartate
NO – nitric oxide
Nogo-A – neurite outgrowth inhibitor A
NRG1 – neuregulin 1
NRP(s) – neural-restricted precursor(s)
NSCISC – National Spinal Cord Injury Statistical Center
N-SC(s) – nerve-derived Schwann cell(s)
NSC(s) – neural stem cell(s)
NSPC(s) – neural stem/progenitor cells
NT – neurotrophin (e.g., NT-3: neurotrophin-3)
O1 – oligodendrocyte marker 1
O4 – oligodendrocyte marker 4
Oct6 – octamer-binding transcription factor 6
OEC(s) – olfactory ensheathing cell(s)
OEG – olfactory ensheathing glia
OMgp – oligodendrocyte-myelin glycoprotein
OPC(s) – oligodendrocyte precursor cell(s)
P₀ – myelin protein zero
P₂X₇ – Purinergic receptor P₂X, ligand-gated ion channel, 7
P₂Y₁₂ – purinergic receptor P₂Y, G-protein coupled, 12
p75 – low-affinity nerve growth factor receptor (aka: p75NTR)
PAN/PVC – polyacrylonitrile / polyvinylchloride
Pax3 – transcription factor paired box 3
PBS – phosphate buffered saline
PDGF – platelet-derived growth factor
PDGFRα – platelet-derived growth factor receptor alpha
PDL – poly-d-lysine
PFA – paraformaldehyde
PI3K – phosphatidylinositol 3-kinase
PLCγ – phospholipase C, gamma
PLP – proteolipid protein
PMP-22 – peripheral myelin protein-22
PN graft(s) – peripheral nerve graft(s)
PNS – peripheral nervous system
PPAR – peroxisome proliferator-activated receptor
PrPc – protease-resistant protein c
PSA-NCAM – polysialylated neural cell adhesion molecule
PTEN – phosphatase and tensin homologue
PVDF – polyvinylidene difluoride
qPCR – quantitative real-time polymerase chain reaction
RGM – repulsive guidance molecule
RM-ANOVA – repeated-measures analysis of variance
RNA – ribonucleic acid
ROS – reactive oxygen species
RST – rubrospinal tract
S100β – S100 calcium-binding protein β
SC(s) – Schwann cell(s)
SCI – spinal cord injury
SCP(s) – Schwann cell precursor(s)
SD – Sprague Dawley
SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM – standard error of the mean
SERT – serotonin transporter
SKP(s) – skin-derived precursor(s)
SKP-SC(s) – SKP-derived Schwann cell(s)
Sox2 – sex determining region Y-related high mobility group-box-2
Sox10 – sex determining region Y-related high mobility group-box-10
SREBPs – sterol regulatory element-binding proteins
Sur1 – sulfonylurea receptor 1
SVZ – subventricular zone
TACE – tumour necrosis factor alpha-converting enzyme
TBST – Tris-buffered saline containing 0.1% Tween-20
TGF-β1 – transforming growth factor beta 1
TH – tyrosine hydroxylase
TLR(s) – Toll-like receptor(s)
tMSC(s) – transdifferentiated mesenchymal stem cell(s)
TNFα – tumour necrosis factor alpha
TRPM7 – transient receptor potential cation channel, subfamily M, member 7
Unc5H2 – uncoordinated 5H2
US – United States
VEGF – vascular endothelial growth factor
WM – white matter
wpi – weeks post-injury
WT – wildtype
YFP – yellow fluorescent protein
Yy1 – Yin yang 1
Acknowledgements

First and foremost I would like to thank my supervisor Wolfram Tetzlaff for giving me the opportunity to do my PhD in his laboratory and for his patience and continued support, both financial and otherwise, throughout my rather lengthy degree. I am also indebted to my supervisory committee members, Drs. Jane Roskams, Fabio Rossi, and Tim O’Connor for their time, patience, and guidance throughout the course of my degree, and I would like to thank my previous/comprehensive committee members, Drs. Janice Eng, Steven Vincent, and Matt Ramer for their valuable input and feedback earlier in my PhD training. Thank you all for making me a better scientist and for encouraging me to expand my technical skills.

I am indebted to our collaborators in the laboratory of Dr. Freda Miller (Toronto). The work herein would not have been possible without the contributions and support of Drs. Miller and Jeff Biernaskie, and I am truly thankful for the culture training provided by both Dr. Biernaskie and Shaalee Dworski during my PhD.

Thank you to the funding agencies that supported me personally, or the projects I worked on, during my graduate training at UBC, including: the Canadian Institutes of Health Research, the Michael Smith Foundation for Health Research, the Stem Cell Network, ICORD, and the faculties of Neuroscience, Medicine, and Graduate Studies (FoGS) at UBC. A special thank you as well, to Rebecca Trainor at FoGS. Your personal support, guidance, and encouragement were instrumental in the completion of this work Rebecca, and I simply cannot thank you enough for pushing me back on track when I wandered off into the woods.

Throughout my degree I was fortunate to be surrounded by highly skilled, intelligent, creative, and supportive people in the Tetzlaff lab. Thank you to Dr. Jason Plemel and Peggy Assinck, whose friendship and support provided a silver lining to even the toughest days of my
PhD. Your words of encouragement and editorial assistance helped make the completion of this work possible Jason. Peggy, thank you for reminding me that helping others is its own reward. I am indebted to you both for helping to keep me sane/motivated throughout my PhD, and I will remember our times together fondly in the future – particularly whenever I smell paraformaldehyde and/or Scotch. I am also very grateful to those who assisted with my training and provided technical assistance on my projects in the Tetzlaff lab, including Clarrie Lam, Jie Liu, Ward Plunet, Loren Oschipok, Casey Shannon, Catherine Hall, Nicole Boeder, Fernando Lucero Villegas, Petra Schreiner, Greg Duncan, Brett Hilton, and Darren Sutherland, and I would like to particularly thank Yuan Jiang, for his amazing technical support and the frequent provision of much needed entertainment/distraction. Thank you as well to the graduate trainees, faculty, and administrative personnel at ICORD who have provided support and encouragement throughout my degree, in particular: Drs. Lowell McPhail, Leanne Ramer, Tom Oxland, Angela Scott, Andrew Gaudet, Brian Kwon, and John Steeves, as well as Tracey Chang, Jeremy Green, Ben Nguyen, Cheryl Niamath, Lisa Anderson, Mark Crawford, Diana Hunter, Jacquelyn Cragg, Jessica Inskip, and Tim Bhatnagar.

To my family I offer my sincere thanks for a lifetime of love, constant encouragement, and unwavering support. Mom, I cannot thank you enough for the years of unconditional love and support that made this accomplishment possible, and I will forever be indebted to you and Sam for giving me a home during the many months it took me to finish this work. Marina, Brianna, Bella, Miguel, Jesus, Anita, Aunt Bonnie, Joan, and Chachi, thank you all for believing in me and reminding me to smile. And finally, thank you to my wife, Monica Rachel Sparling, for her continued patience, love, encouragement, and support throughout my PhD. You shared the weight of this work with me Mon, and for that I will always be immeasurably grateful.
Dedication

To my beautiful wife, Monica Rachel Sparling. I’m sorry this took so long.
Chapter 1:

General Introduction
1.1 Overview of introduction

Spontaneous repair processes generally fail following injury to the adult mammalian central nervous system (CNS), resulting in permanent functional deficits, the variety and severity of which depend on the location and extent of neurologic damage. In the context of spinal cord injury (SCI), this commonly translates into life-long deficits in motor, sensory, and/or autonomic functions below the level of injury, as well as a high frequency of related secondary complications (e.g., urinary tract infections, pressure ulcers, etc.); the combination of which is often devastating, not only to a person’s physical well-being, but also to his/her quality of life and life expectancy (Dijkers, 2005; Dietz and Curt, 2006; Strauss et al., 2006; Grossman et al., 2012; NSCISC, 2012). Although considerable advances have been made in the surgical and medical management of SCI and related secondary complications (Knoeller and Seifried, 2000; Lifshutz and Colohan, 2004; Donovan, 2007; Parent et al., 2011), at present, there exists no therapeutic intervention proven effective in restoring the loss of neurologic functions following SCI (Harrop et al., 2012; Hug and Weidner, 2012; Illis, 2012; Lammertse, 2013). Towards that goal, a wide variety of promising therapeutics are currently being investigated in pre-clinical animal studies (see reviews by: Kwon et al., 2011a; Kwon et al., 2011b; Tetzlaff et al., 2011) as well as human clinical trials (see reviews by: Gensel et al., 2011; Harrop et al., 2012; Wilcox et al., 2012; Lammertse, 2013).

One promising potential therapy for SCI treatments involves the transplantation of Schwann cells (SCs) into the injured spinal cord. SC transplantation to treat SCI is particularly attractive because those cells have demonstrated functional efficacy in a wide variety of animal models of SCI and they can be generated from autologous tissue sources in the adult; thereby
obviating the need to use potentially harmful immunosuppressive drugs to prevent the rejection of transplanted cells following clinical application (reviewed in: Bunge and Pearse, 2003; Oudega et al., 2005; Oudega and Xu, 2006; Tetzlaff et al., 2011). Indeed, the potential of these cells to improve recovery from SCI has prompted multiple clinical trials to examine the safety and potential benefits of SC transplantation in humans with SCI (Tator, 2006; Saberi et al., 2008; Saberi et al., 2011; Talan, 2012; Xian-Hu et al., 2012). There remains however, one major drawback to the clinical application of autologous SC therapies, as these cells are typically generated via the excision of sural nerve in humans (nerve-derived SCs; N-SCs) and this procedure not only requires an additional surgery, but also results in a permanent peripheral sensory deficit and carries the risk of painful neuroma formation (Wolford and Stevao, 2003; Hood et al., 2009). Although the loss of sensation in the periphery may be well worth any gain in central functions, particularly given that the negative effects of peripheral nervous system (PNS) injury may go unnoticed in patients with existing SCI-related sensory deficits, it is obviously not ideal to injure the PNS in order to treat the CNS, particularly given that one aim of SC transplantation is to ameliorate the loss of sensation caused by SCI. This situation would clearly be improved if SCs could be generated from autologous tissue sources other than peripheral nerve, and indeed this can be done, as alternative sources of SCs have now been found in adult rodent and human tissues.

Arguably the most promising alternative source of SCs that has been identified to date are the skin-derived precursors (SKPs); a resident stem cell found in adult mammalian skin (Biernaskie et al., 2009). Under appropriate conditions, isolated SKPs can be differentiated into SCs (SKP-SCs) in culture, and preliminary work with those cells demonstrated that they display
SC-like morphology, label with many of the antibodies typically associated with N-SCs, and show characteristic SC myelination of both PNS and CNS axons (Biernaskie et al., 2006; McKenzie et al., 2006). In light of those characteristics, we hypothesized that SKP-SCs would be a suitable alternative to N-SCs for therapeutic transplantation following SCI. To test that hypothesis, we began by assessing the benefits of SKP-SC transplantation following thoracic contusion in rats (Chapter 2); a model that has been used repeatedly to demonstrate the efficacy of N-SC transplantation after SCI (Tetzlaff et al., 2011). The results of that study demonstrated that SKP-SCs provide many of the same benefits typically associated with N-SC transplantation after SCI, including a modest, but significant improvement in hindlimb motor function. In addition, we noted that the SKP-SCs appeared to have advantages over N-SCs transplanted under similar conditions in terms of the degree of reactivity they elicit from nearby astrocytes and their abilities to migrate/integrate into astrocyte-rich host tissue and support the growth of certain axon populations, particularly across the distal graft-host interface. In light of the fact that cultured SKP-SCs are newly generated SCs, and in recognition of evidence that less mature SCs (i.e., Schwann cell precursors harvested from embryonic nerve) display similar advantages over N-SCs harvested from postnatal nerve (Woodhoo et al., 2007), we hypothesized that SKP-SCs may simply represent a population of less mature SCs compared to the N-SCs harvested from peripheral nerve. As such, and in light of our previous findings, we also hypothesized that SKP-SCs have advantages over N-SCs in terms of their interactions with astrocytes and their ability to support axonal growth, and we predicted that those advantages would endow the SKP-SCs with greater efficacy as a treatment for SCI.
To test those hypotheses, we conducted two concurrent studies comparing SKP-SCs and N-SCs harvested from neonatal rat tissue side-by-side both in vitro and in vivo. The first of those studies examined the SKP-SCs as a treatment for incomplete cervical SCI and directly compared the behaviour and reparative efficacy of SKP-SCs and N-SCs transplanted into the injured cervical spinal cord in an effort to determine whether either cell type had an advantage in terms of their efficacy as a treatment for SCI. The second study examined the interactions of SCs from either source with astrocytes in vitro and astrocyte-rich host tissue in the injured spinal cord to more precisely delineate the advantages of SKP-SCs over N-SCs, and assessed the expression of a variety of proteins and genes related to SC development in an effort to determine whether the SKP-SCs truly represent a less mature SC phenotype than their nerve-derived counterparts.

Consistent with our hypotheses, the SKP-SCs proved to be efficacious as a treatment for incomplete cervical SCI. However, contrary to our hypotheses, we found few significant differences between SKP-SCs and N-SCs, either in vitro or following transplantation into the injured spinal cord, which indicated that SKP-SCs do not have an advantage over N-SCs with respect to their suitability or efficacy in CNS repair when those cell types are both generated from neonatal tissue sources. Furthermore, SKP-SCs and N-SCs were not found to differ in terms of their expression of any of the characteristic SC marker proteins/genes that we examined, and our examination indicated that cells from either source were relatively immature and shared highly similar phenotypes in terms of their interactions with astrocytes overall.

Thus, our findings suggest that SKP-SCs may possess a phenotype that is more similar to that of N-SCs generated from neonatal, as opposed to adult, peripheral nerve. Our findings also support the notion that SKP-SCs are a suitable alternative to N-SCs for transplantation-based
repair of the injured CNS, and add to a growing body of evidence suggesting that SKP-SCs may have therapeutically relevant advantages over their counterparts generated from adult nerve. Although more work comparing SKP-SCs and N-SCs from adult rodent and human tissue sources is required to confirm the present findings, and definitively establish the suitability of SKP-SCs for clinical applications, this work carries important implications for the potential clinical translation of SKP-SCs as a cellular therapy for SCI.

To facilitate a detailed discussion of the research described in Chapters 2 through 4, the remainder of this introductory chapter provides a rather thorough review of the major topics pertinent to the experiments presented herein. I begin by discussing SCI with a particular focus on the need for effective treatments to promote neurological repair and functional recovery. Next I describe the pathophysiology of SCI including a detailed account of secondary injury and the response to injury displayed by various CNS resident cell types. Following that I delve into a general discussion regarding therapeutic strategies for CNS repair, with an emphasis on cellular therapies for SCI. Next I examine the topic of SCs including a detailed discussion of their development, their functions in the intact and injured PNS, the benefits and limitations of N-SC transplantation as a treatment for CNS injury/disease, and finally the drawbacks of using peripheral nerve as a source for autologous SCs for therapeutic clinical applications. At that point I critically analyze the alternative (i.e., non-nerve) sources of autologous SCs, other than SKPs, that have been suggested as potentially suitable replacements for N-SCs in the clinic. And finally, I review what was known about the SKPs and SKP-SCs prior to the initiation of the present work; which leads rather directly into the overview of the experiments conducted and hypotheses tested in the present work.
1.2 Spinal cord injury

1.2.1 Causes of SCI

SCI is extremely heterogeneous in humans, as a variety of both traumatic (e.g., motor vehicle accidents) and non-traumatic (e.g., ongoing infection/disease) events can cause damage to spinal cord tissue, and the functional ramifications of that damage depend on multiple factors including the pattern of pathology associated with a particular mechanism of injury, as well as the precise location of the injury and the extent of tissue damage (Kirshblum et al., 2002; Norenberg et al., 2004). The animals models used to test the potential therapeutic benefit of SKP-SC transplantation in the present work (thoracic contusion and dorsolateral funiculus crush) are traumatic in nature, and thus traumatic SCI is the focus of this discussion and the term SCI is used throughout this work to refer to traumatic SCI unless otherwise stated.

In humans, the frequency of different causes of SCI varies according to geographical location, often reflecting cultural and socioeconomic differences among nations and regions (Farry and Baxter, 2010; Cripps et al., 2011), but worldwide SCI appears to disproportionately affect males as compared to females, as the estimated incidence in males across multiple studies is 2-4 times that in females (Dryden et al., 2003; Wyndaele and Wyndaele, 2006; Turner et al., 2009; Couris et al., 2010; NSCISC, 2012). The exception to the latter appears to be in the elderly population where men and women show similar rates of injury, mostly due to falls at home (Spivak et al., 1994). In North America, as in most developed nations, the most common cause of traumatic SCI has historically been motor vehicle accidents, but recent reports indicate that work accidents and falls at home are becoming more prevalent as the median age of the general population rises (Tator, 1995a; Dryden et al., 2003; Pickett et al., 2006; Turner et al., 2009;
Couris et al., 2010; Farry and Baxter, 2010; Cripps et al., 2011; NSCISC, 2012). These types of incidents tend to result in vertebral dislocations and fractures of the spinal column, which represent the most common injuries observed in the clinic, where vertebral dislocations (with and without fracture) occur in 29-45% of cases and burst fractures occur in 30-48% of cases (Sekhon and Fehlings, 2001; Pickett et al., 2006). Vertebral dislocations and fractures most often result in contusion and some degree of compression and/or distraction of the spinal cord, and although laceration and even full transection of the spinal cord can occur as a result of severe fracture-dislocation or foreign bodies (e.g., knives and bullets) traversing the vertebral canal, those primary mechanisms of injury are much less common clinically (Bunge et al., 1993; Dumont et al., 2001; Norenberg et al., 2004).

1.2.2 Relating spinal cord anatomy to functional losses due to SCI

With the exception of functions subserved by the cranial nerves that arise directly from the brain or brainstem, all communication between the brain and peripheral organs and tissues occurs via the spinal cord. The spinal cord contains numerous ascending (sensory) and descending (motor) tracts of axons that run along its length in the white matter at specific locations in the dorsoventral-mediolateral axes (i.e., transverse plane) and carry information between structures in the brain and each level of the spinal cord (Guertin, 2012). Sensory and motor axons enter/exit the spinal cord via the sensory and motor nerve roots that comprise the spinal nerves, which are continuous with the peripheral nerves that innervate specific organs and tissues at each vertebral level (Guertin, 2012). At the cervical level, the spinal nerves innervate the head, neck, shoulders, chest, upper limbs and respiratory organs, whereas at the thoracic level the spinal nerves primarily innervate the trunk and its organs, and at the lumbar and sacral levels
they innervate the lower limbs and pelvic organs (McDonald, 1999). Thus the spinal cord is anatomically organized to enable communication between specific regions of the brain and specific peripheral organs/tissues at each vertebral level, but it is important to note that the spinal cord is not a passive conduit for that communication, but rather an active participant in the integration and modulation of inputs from descending supraspinal pathways and peripheral afferents (Flynn et al., 2011).

In addition to the long ascending and descending axon tracts in the white matter, the neuronal content of the spinal cord is largely composed of interneurons that form circuits within the gray matter of each spinal segment and propriospinal interneurons that give rise to intraspinal axons that project via the white matter to the gray matter in other spinal segments, sometimes over substantial distances (Conta and Stelzner, 2009). The intraspinal network of interneurons, including propriospinal neurons, plays a critical role in motor reflexes, voluntary movement, and sensory processing (reviewed by Flynn et al., 2011). Gray matter interneurons form simple spinal reflex pathways that enable involuntary autonomic and somatic motor responses to peripheral stimuli in the absence of supraspinal input, but they also form more complex networks known as central pattern generators (CPGs), that generate patterned or rhythmic responses in multiple muscle groups and provide the basic motor commands for more complex behaviours such as locomotion, scratching, micturition and even ejaculation (Guertin and Steuer, 2009; Flynn et al., 2011; Guertin, 2012; Courtois et al., 2013). Unlike simple reflexes, CPG-driven functions are subject to conscious/voluntary control, as locomotor CPG activity is induced and modulated by supraspinal input, although it is also modulated directly by primary afferent input, which enables the automatic adjustment of CPG-generated motor output in response to sensory feedback
Interneurons in the gray matter of the cervical and lumbar enlargements are believed to form motor CPGs that generate the basic motor patterns underlying rhythmic forelimb/hindlimb or upper/lower limb movements, respectively, in all vertebrates including humans (Dietz et al., 1994; Kiehn and Butt, 2003; van Hedel and Dietz, 2010; Harkema et al., 2011; Guertin, 2012). Although those circuits are normally under supraspinal control, there is evidence from many species, including humans, that propriospinal connections couple the cervical and lumbar CPGs and thus enable the unconscious coordination of forelimb/hindlimb or arm/leg movements during locomotion to be achieved largely at the spinal level (Juvin et al., 2005; Dietz and Michel, 2009; Zehr et al., 2009; Juvin et al., 2012).

In adult humans, as with all adult mammals, spontaneous repair of the injured CNS generally fails, so SCI causes permanent damage to both ascending and descending white matter tracts as well as the interneurons and propriospinal axons of the gray matter, leading to permanent neurological impairments that affect motor, sensory, and autonomic functions. As a result of the anatomical organization of the white matter tracts, damage to specific tracts in the spinal cord disrupt the connection between the brain and portions of the periphery innervated by that tract below the level of injury (Blight, 2002). Thus, an injury at the level of the 4th cervical vertebra (C4) disrupts communication between the brain and all peripheral structures innervated by the injured spinal tract(s) from C5 down, including thoracic, lumbar, and sacral levels, but does not disrupt communication between the brain and peripheral organs/tissues innervated by spinal nerves at levels above the injury (e.g., C2). In addition to the effects of damage to axon tracts in the white matter, SCI often results in damage to the gray matter, causing the loss of interneurons involved in local reflex pathways, CPGs, and/or propriospinal connections between
spinal segments, which lead to functional deficits in reflexes, patterned motor responses, and/or the coordination of responses, respectively (Flynn et al., 2011). Even if a CPG remains intact, injury in rostral segments can cut off the supraspinal input required to induce or properly modulate its activity, thereby disturbing or eliminating voluntary control of CPG-dependent patterned activities such as locomotion. Although there is evidence to support the existence of CPGs in humans, their role in locomotion remains hotly debated, as it is thought that supraspinal input may play a more extensive part in the regulation of locomotion in man compared with lower vertebrates (Dietz and Michel, 2009; Guertin, 2012).

1.2.3 The outcome of SCI in humans

In humans the neurological impairments caused by SCI often include paralysis, stiffness or spasticity of the skeletal muscles, lost or abnormal sensation (anesthesia, hypoesthesia, paresthesia, or dysesthesia) across multiple sensory modalities (e.g., touch, temperature, vibration), persistent neuropathic pain, as well as deficits in cardiovascular, respiratory, bowel, bladder, and/or sexual functions (Levi et al., 1995c; Watanabe et al., 1996; Stiens et al., 1997; Blight, 2002; Winslow and Rozovsky, 2003; Anderson, 2004; Gustin et al., 2010; Popa et al., 2010; Teasell et al., 2010; Zeilig et al., 2012). The majority of traumatic SCI occurs at the cervical level and thus impairs function in the pelvic organs, trunk, and upper and lower limbs (i.e., tetraplegia / quadriplegia), while the remaining injuries occur at the thoracic, lumbar or sacral vertebral levels and result in lower limb and trunk impairments only (i.e., paraplegia) (Farry and Baxter, 2010; NSCISC, 2012). The degree of functional loss varies substantially according to the severity of neurological damage, but a general distinction is often made between ‘complete’ injuries, which are characterized by a total loss of sensory and motor functions below
the level of damage, and ‘incomplete’ injuries in which some degree of residual neurological function is maintained. The most severe neurologic impairments usually occur in individuals with complete tetraplegia resulting from high cervical (C1-C4) SCI, as in addition to the complete loss of motor and sensory function of all limbs, and a range of severe autonomic disturbances, these individuals often require assistance breathing (e.g., mechanical ventilation or phrenic nerve pacing) due to the loss of control of the muscles required for respiration (Winslow and Rozovsky, 2003). Most individuals with SCI have less severe neurologic impairments, as complete tetraplegia is relatively rare compared to all other types of SCI combined, only occurring in 16% of new injuries in the United States (US) since 2005 for example, and only a fraction of those cases involve injury at high cervical levels (NSCISC, 2012). However, complete neurologic recovery is rare (<1%) after any trauma to the spinal cord, so most people with SCI will suffer some degree of long-term disability, and many require assistive / adaptive devices (e.g., wheel chair or vehicular hand controls), assistance in daily living (e.g., homecare), and continued/frequent medical supervision (Dryden et al., 2004; Turner et al., 2009; Guilcher et al., 2010; DeVivo et al., 2011; NSCISC, 2012).

The neurological impairments caused by SCI are often directly associated with a host of secondary health complications (e.g., deep vein thrombosis, urinary tract infections, pressure sores / ulcers and respiratory infections), the variety and severity of which depend largely on the degree of disability, and particularly the severity of autonomic dysfunctions (Levi et al., 1995c; Winslow and Rozovsky, 2003; Dryden et al., 2004; Christie et al., 2011; DeVivo and Farris, 2011). These complications contribute substantially to SCI-related health issues, resulting in increased need for medical attention and rehospitalization long after initial discharge, and they
are often the cause of mortality in the SCI population (Levi et al., 1995b; Dryden et al., 2004; Shavelle et al., 2006; Strauss et al., 2006; Christie et al., 2011; DeVivo and Farris, 2011; NSCISC, 2012).

In developed nations, survival after SCI, particularly during the first year or two post-injury, has improved dramatically since the 1940s due to advances in emergency medical services and the treatment of SCI and related secondary complications (see below), and yet individuals living with SCI continue to have a reduced life expectancy compared to their age-matched uninjured peers in the general population (Frankel et al., 1998; Imai et al., 2004; Shavelle et al., 2006; Strauss et al., 2006; Farry and Baxter, 2010). Mortality rates are significantly higher during the first year or two after SCI, particularly for people with more severe injuries and those injured at an older age (Shavelle et al., 2006; Strauss et al., 2006; NSCISC, 2012), but those rates remain elevated in the SCI population compared to the general population, even after that critical period. For example, a 20 year old male with paraplegia who survives at least a year post-injury is expected to live to reach 65 years of age, whereas a person in the general population without a SCI has a life expectancy of 79 years (NSCISC, 2012). Had the same 20 year old received an injury causing high tetraplegia with ventilator dependence, he would only be expected to reach the age of 45 (NSCISC, 2012).

In addition to the negative impact of SCI on physical health and well-being, the combination of neurological impairment, persistent pain, and secondary health complications can be devastating in terms of psychological health, as individuals with SCI are at increased risk for depression and anxiety and often report lower perceived quality of life than the general population (Davidoff et al., 1992; Evans et al., 1994; Levi et al., 1995b; Elliott and Frank, 1996;
Dijkers, 1997; Krause, 1998; Tate et al., 2002; Sakakibara et al., 2009; Shin et al., 2012). This is particularly the case for individuals with more severe injuries and older people with SCI who tend to require more physical assistance (Gerhart et al., 1993; Evans et al., 1994; Shin et al., 2012). For non-disabled individuals, the mental and emotional toll of SCI may be difficult to fully comprehend, but the culmination of those effects is clearly and sadly demonstrated by the fact that a person with SCI is five times more likely to commit suicide than someone in the general populace (Dijkers et al., 1995).

Beyond the physical, mental, and emotional burdens, there is also a high financial cost to living with SCI. In addition to expenses related directly to compensating for physical disabilities (e.g., assistive / adaptive devices, home renovations/moving costs to ensure accessibility, etc.), individuals with SCI also tend to require more frequent and longer hospitalizations, greater medical attention in general, more prescription medication, and significantly more homecare service compared to people in the general population (Levi et al., 1995b; Dryden et al., 2004; Munce et al., 2009; Guilcher et al., 2010; DeVivo and Farris, 2011). Depression, anxiety, sleep disturbance and fatigue are commonly cited as reasons for seeking medical attention, so clearly the psychological toll of SCI contributes to increased health care utilization in this population (Levi et al., 1995c; Levi et al., 1995b; Dryden et al., 2004). Recurring or persistent secondary complications and hospital admissions represent disruptions to daily life, which are costly both financially and socially, and may negatively impact quality of life (Menter et al., 1991; Johnson et al., 1996; Krause, 1998). SCI often has a negative effect on employment and income (Krause et al., 2011) and tends to disproportionately affect individuals from low income families (at least in the US) (Turner et al., 2009). As such, many people living with SCI have neither the financial
means nor sufficient medical insurance coverage to accommodate SCI-related medical, homecare, and living expenses and a large proportion of those individuals are forced to rely on publicly funded health care and informal homecare by friends and relatives (Turner et al., 2009). The situation is far more dire for individuals in undeveloped nations (e.g., Nigeria, Zimbabwe, and South Africa), where most patients die either before they reach a treatment centre or within the first year post-injury (Cripps et al., 2011).

1.2.4 The global cost of SCI

SCI is a global health problem that afflicts millions of people worldwide and represents a substantial burden on society and the global economy. According to data from studies conducted worldwide between 1995 and 2005, the incidence of SCI varies considerably from country to country, and the global incidence of SCI ranges from 10.4-83 cases per million inhabitants per year, depending on the country in question and the methods of estimation used (Wyndaele and Wyndaele, 2006). Excluding those who die prior to hospitalization, a more recent estimate places the annual incidence of SCI at about 40 cases per million people, or approximately 12,000 new cases each year in the US (NSCISC, 2012). Similar values have been found in Canada, where the Rick Hansen Institute reports about 1,400 new cases of traumatic SCI (not resulting in death prior to hospitalization) are estimated to occur each year; which translates to 41 cases per million inhabitants per year (Farry and Baxter, 2010; Noonan et al., 2012).

Among developed nations, the average estimated prevalence of SCI is 485 cases per million inhabitants based on SCI registry data (Wyndaele and Wyndaele, 2006). Assuming that a similar rate applies worldwide, and given a current world population of roughly 7 billion (http://www.census.gov/popclock/), that prevalence translates into nearly 3.5 million people
living with SCI on our planet. According to estimates from a recent household survey by the Christopher and Dana Reeve Foundation, nearly 1.3 million of those individuals live in the US alone (Turner et al., 2009), although prevalence rates based on SCI registry and hospitalization data are much lower, with an upper range of approximately 327,000 persons (NSCISC, 2012). In Canada the most recent estimate of the prevalence of SCI is ~85,000 persons, and nearly half of those cases occurred as a result of non-traumatic causes, such as diseases, infections, or tumours (Farry and Baxter, 2010; Noonan et al., 2012). In contrast, the data from the Reeve Foundation showed that only 18% of patients with SCI in the US did not directly attribute that injury to physical trauma (Turner et al., 2009).

The estimated combined annual cost of health care and living expenses for individuals with SCI varies greatly according to the level and severity of injury, as do the estimated lifetime costs directly attributable to SCI (Cao et al., 2011; DeVivo et al., 2011). According to the National Spinal Cord Injury Statistical Center (NSCISC; Birmingham, Alabama), the average estimated expenses in the first year after injury range from $334,000 for individuals with incomplete motor function at any level to just over $1 million for those with high tetraplegia (C1-C4), and the estimated costs each subsequent year range from $41,000 to $178,000 for those same groups; all values in Feb 2012 US dollars (NSCISC, 2012). The NSCISC also provides the most recent estimates of the lifetime costs for an individual with SCI, which range from $1.5 million (incomplete motor function at any level) to $4.5 million (C1-C4 tetraplegia) for someone injured at 25 years of age, and $1 million to $2.5 million for those same groups if the individual is injured at 50 years of age; all values in Feb 2012 US dollars (NSCISC, 2012). Importantly, those figures do not include any indirect costs (e.g., lost wages, benefits, and/or productivity),
which costs individuals with SCI an estimated $69,000 (Feb 2012 US dollars) per person per year, though that value varies considerably depending on the level and severity of injury, education, and pre-injury employment history (NSCISC, 2012). In 2009, SCI was estimated to cost the US health care system $40.5 billion (2009 US dollars) annually (Turner et al., 2009). Based on an estimated prevalence of 3.5 million people with SCI worldwide (Wyndaele and Wyndaele, 2006) and the fact the US is estimated to account for only perhaps 37% of that population (Turner et al., 2009), the extrapolated health care cost for SCI worldwide is $109 billion (2009 US dollars) annually. That estimate is unlikely to accurately represent the worldwide SCI-related health care expenditures, as the availability and cost of SCI-related health care varies substantially from nation to nation, but it does drive home a significant point – SCI represents a massive economic burden to people and societies around the globe.

1.2.5 A brief history of the treatment of SCI in humans

SCI has obviously been occurring in humans since the dawn of the species, but the first known medical documentation of SCI cases occurred in the Edwin Smith papyrus (Hughes, 1988), which is an ancient Egyptian surgical treatise that dates to roughly 1600 BCE (Allen, 2005). In that ancient text, SCI was described as “an ailment not to be treated” (Hughes, 1988), and sadly, that viewpoint continued to dominate the care of patients with SCI for about 3,500 years thereafter; during which time SCI was generally considered a death sentence (Donovan, 2007). During most of that period, the only intervention that was relatively widely adopted for SCI was non-surgical (i.e., closed) traction to treat spinal fractures and dislocations; a method originally championed by Hippocrates (460-360 BCE), although he did not actually advocate it when fracture was coupled with paralysis (Richards, 1968; Knoeller and Seifried, 2000; Lifshutz
and Colohan, 2004). The first recorded surgical intervention for SCI was the removal of bone fragments impinging on the spinal cord, a procedure performed by Paulus of Aegina (625-690 CE) over 2,000 years after the ancient Egyptians first described SCI (Knoeller and Seifried, 2000; Lifshutz and Colohan, 2004). However, that and other surgical techniques generally failed to gain wide acceptance over the next 1,300 years, as spinal column surgery was extremely painful, often failed, and frequently resulted in death due to infection (Knoeller and Seifried, 2000; Donovan, 2007). Between the late 19th and early 20th centuries, the ease and safety of all surgical interventions were drastically improved due to the application (and refinement) of aseptic techniques, general anesthetics, and antibiotics in medicine, and these advances prompted increased efforts to develop surgical interventions to treat spinal trauma (Donovan, 1994; Knoeller and Seifried, 2000; Donovan, 2007). With increased surgical efforts, came improved surgical techniques, and combined with improvements in imaging techniques (i.e., the development of computerized tomography [CT] and magnetic resonance imaging [MRI]) and spine surgery instrumentation (e.g., fusion rods, plates, and pedicle screws) these advances eventually led to the development of the safe and effective procedures for stabilizing the fractured spine and decompressing the injured spinal cord that are used today (Knoeller and Seifried, 2000; Donovan, 2007).

In the early 20th century, medical advances had yet to impact the treatment of SCI, so the prospect of surviving a SCI remained extremely poor. For example, in World War I, 80% of American soldiers with traumatic SCI died within the first two weeks, and only 10% survived to 1 year after injury (Guttmann, 1946). However, the 1940s heralded marked improvement in survival after SCI, largely due to the application of antibiotics, but also the introduction of SCI
management units dedicated to the prevention and management of secondary complications and
comprehensive rehabilitation, and this trend in improved survival has continued since then, with
more recent advances in emergency medical services and the development of effective surgical
interventions for spinal fractures and dislocations, in the 1960s and 1970s respectively
(Whiteneck et al., 1992; Samsa et al., 1993; Knoeller and Seifried, 2000; Donovan, 2007). The
improved survival of patients with SCI meant that for the first time in history there was a
growing population of individuals with chronic SCI and therefore a growing need for long-term
care and effective treatment of this condition, and by the 1980s, recognition of the latter finally
started spawning large-scale clinical trials for interventions aimed at treating the injured spinal
cord itself, rather than merely the bones of the spinal column.

Over the preceding century, the testing of experimental therapeutics for SCI had expanded substantially, fuelled by growing knowledge of neuroanatomy and neurobiology, and
new insights into the underlying pathology of SCI from animal research (Lifshutz and Colohan,
2004). Promising results from work in animals with SCI prompted clinical testing of a wide
variety of experimental treatments throughout that period (e.g., enzymes to dissolve scars,
electrical stimulation, nerve transplants, omental transposition, and spinal cord cooling), but
most were only examined in a single patient or a group of patients treated by a single clinician,
and despite the fact that many of those experiments reported ‘successful’ results, none were ever
proven efficacious or widely adopted (Tator, 2006). One exception to that was the use of
corticosteroids, which became a ‘standard of care’ for acute SCI by the 1980s, despite a lack of
firm clinical evidence that it was beneficial (Lammertse, 2013).
The late 20th century saw a gradual shift in medicine towards evidence-based practice, which emphasizes that empirical evidence from properly conducted clinical research should be the cornerstone of clinical decision making, rather than subjective clinical experience (Sackett et al., 1996; Howick, 2011). This shift was facilitated by many factors, not the least of which was the adoption of new premarket approval regulations for novel pharmaceutical drugs by the US Food and Drug Administration (FDA) in 1962 (Kaplan et al., 2011). Those new regulations led to the adoption of randomized controlled clinical trials, which were mandatory to prove the effectiveness of new drugs in order to attain FDA approval to market a drug as a treatment for a specific condition (Kulynych, 1999). Over time the use of randomized controlled trials became the ‘gold standard’ for establishing efficacy in all medical research, and its application expanded from drug testing to the evaluation of surgical and even diagnostic procedures (Kaplan et al., 2011).

In the field of SCI, the clinical trial era began in February of 1979 with the enrolment of the first patient in the multicentre double-blind randomized controlled trial of methylprednisolone (Bracken et al., 1984; Lammertse, 2013). However, it wasn’t until the mid-1980s and beyond that the large-scale, randomized, placebo-controlled trials began producing data regarding the efficacy of interventions aimed at treating the underlying neuropathology of SCI; e.g.: methylprednisolone (Bracken et al., 1990; Bracken et al., 1997) and GM-1 ganglioside (Geisler et al., 1991; Geisler et al., 2001). This marked an important transition in SCI clinical research, as the focus had now largely shifted from promoting survival and minimizing physical trauma in SCI patients to promoting neuroprotection and regeneration using pharmacological / biological interventions directed at the underlying neuropathology responsible for functional
deficits. During these early trials the transplantation of tissue and cells was still in its infancy, and remained largely limited to single case reports or small non-randomized exploratory trials that mainly addressed treatment safety (i.e., Phase I clinical trials) (for examples see: Falci et al., 1997; Wirth et al., 2001; Tadie et al., 2002; Rabinovich et al., 2003; von Wild and Brunelli, 2003; Cheng et al., 2004; Feron et al., 2005; Knoller et al., 2005; Park et al., 2005; Schwartz and Yoles, 2005); with the exception of the transplantation of autologous activated macrophages, which was the first cell-based therapy subjected to randomized controlled trial in studies initiated in 2003 (Lammertse et al., 2012). In the meantime, a vast amount of preclinical research in animal models of SCI continued to increase knowledge regarding the underlying mechanisms of injury and suggest novel treatments for clinical application, prompting ever increasing numbers of clinical trials in the field of SCI (Tator, 2006; Lammertse, 2013).

1.2.6 The current state of treatment for SCI in humans

To date, there have been an impressive number of clinical trials for treatments aimed at improving neurological recovery following SCI (e.g.: Bracken et al. 1990; Bracken et al. 1997; Cardenas et al. 2007; Casha et al. 2012; Dobkin et al. 2006; Fehlings et al. 2011; Fehlings et al. 2012a; Geffner et al. 2008; Geisler et al. 2001; Kapadia et al. 2011; Karamouzian et al. 2012; Kumar et al. 2009; Lammertse et al. 2012; Levi et al. 2009; Lima et al. 2010; Lorenz et al. 2012; Pal et al. 2009; Pitts et al. 1995; Pointillart et al. 2000; Popovic et al. 2011; Rabinovich et al. 2003; Saberi et al. 2011b; Shapiro et al. 2005; Tadie et al. 2003; Tator et al. 1987; Triolo et al. 2012; Vaccaro et al. 1997; Vale et al. 1997; Wu et al. 2012; Yang et al. 2012; Yoon et al. 2007; Zariffa et al. 2012). However, the testing of many of those potential treatments remains in the preliminary stages (i.e., Phase I or II clinical trials), where small groups of patients are exposed
to a treatment to establish safety and appropriate dosing regimens, as relatively few treatments have been subjected to the kind of testing that is necessary to establish clinical efficacy (e.g., Phase III clinical trials using large randomized placebo-controlled trials; or large non-randomized prospective observational studies). Of those treatments that have undergone adequate testing to definitively determine efficacy, most have failed to demonstrate sufficient neurological benefit to warrant their adoption as a standard of care for the treatment of SCI (Bracken et al., 1990; Bracken et al., 1997; Pointillart et al., 2000; Geisler et al., 2001; Tadie et al., 2003; Lammertse et al., 2012; Yang et al., 2012). The corticosteroid methylprednisolone was thought to be a rare exception to that, as initial reports from randomized placebo-controlled trials indicated a modicum of therapeutic efficacy (Bracken et al., 1990; Bracken et al., 1997), which led to rapid and widespread clinical application, but clinical use of methylprednisolone has subsequently declined significantly due to continued scrutiny of trial evidence and research demonstrating a high risk of serious health complications at the effective dosage used to treat SCI (Hurlbert, 2000; Pointillart et al., 2000; Hugenholtz, 2003; Hurlbert and Hamilton, 2008).

Of all of the treatments tested so far, only surgery and rehabilitation approaches have yielded sufficient safety and therapeutic efficacy (i.e., evidence of neurological or functional recovery) in large enough groups of patients to justify their adoption as standards of care following SCI. Early (<24 hours post-SCI) surgical stabilization and decompression of the spinal cord was recently shown to be associated with significant neurological improvement at 6 month follow-up in patients with cervical SCI regardless of the completeness of injury (Fehlings et al., 2012b). In contrast, although physical rehabilitation using manual/automated activity-based rehabilitation (Thomas and Gorassini, 2005; Wirz et al., 2005; Dobkin et al., 2006; Harkema et
al., 2012b; Harkema et al., 2012a; Lorenz et al., 2012; Zariffa et al., 2012) with/without functional electrical stimulation (Ladouceur and Barbeau, 2000; Kapadia et al., 2011; Popovic et al., 2011) has been found to improve neurological function in patients over time following injury, those effects are generally only significant for patients with incomplete injuries (i.e., some preservation of function). Thus, although the medical, surgical, and rehabilitative treatments for SCI and related secondary complications advanced considerably during the late 20\textsuperscript{th} century and beyond, standard medical practice following SCI has largely remained focused on the same approaches: the prevention and management of secondary complications to maintain health, surgical decompression and stabilization to minimize damage, and physical rehabilitation to optimize recovery (Mothe and Tator, 2012). And so, despite advances in the treatment of SCI over the last 30-40 years, neuroprotection and neurological repair (i.e., regeneration) remain quite limited, and SCI continues to cause lifelong disability and neurological dysfunction.

Although that summary appears quite bleak and it is true that no regenerative therapy has proven to be beneficial in human SCI to date (Fawcett, 2002; Illis, 2012), it is important to keep in mind that there are many therapeutic options that have yet discovered, let alone adequately tested for efficacy following SCI. Thus, although many treatments have been proven ineffective, a variety of potential treatments have demonstrated preliminary signs of efficacy in early (Phase I/II) clinical trials and currently await testing in large randomized controlled trials designed to definitively determine efficacy (e.g.: Casha et al. 2012; Fehlings et al. 2011; Kumar et al. 2009; Lima et al. 2010; Saberi et al. 2011b; Yoon et al. 2007). In addition, there are many clinical trials aimed at neuroprotection and/or neurological repair after SCI that are currently in the planning, recruiting, or analysis phases (Fehlings et al., 2012a; Talan, 2012; Tator et al., 2012; Wilcox et
al., 2012), any one of which may also provide a new standard of care for the treatment of SCI in humans.

1.3 The pathophysiology of SCI

The long-term pathological consequences of traumatic SCI are the result of two distinct, yet related, processes commonly referred to as primary and secondary injury. Primary injury refers to tissue damage that is directly attributable to the initial trauma to the spinal cord, whereas secondary injury refers to a cascade of pathophysiological events at the tissue, cellular, and molecular levels (e.g., ischemia, inflammation, oxidative stress, etc.) that are triggered by primary injury and contribute to tissue damage over time following injury (Tator and Fehlings, 1991; Tator, 1995b). The only treatment that can be directed at primary injury is prevention, as there is no intervention that can stop trauma from occurring when external forces are applied directly to the spinal cord. In contrast, secondary injury mechanisms involve protracted tissue damage that accumulates over time, and may therefore be ameliorated by interventions designed to counteract the underlying pathological processes. This is not as straightforward as it sounds however, as there are a large number of secondary injury mechanisms at work following SCI and many of those mechanisms are inter-related.

1.3.1 Primary injury

Primary injury mechanisms typically include contusion (i.e., impact), compression, distraction (i.e., forcible stretching of the spinal column in the axial plane), and laceration of the spinal cord (Dumont et al., 2001). In humans, SCI often involves multiple primary injury mechanisms. For example, contusions resulting from spinal fractures are always associated with
some degree of compression whether transient or persistent, whereas dislocations of the spinal column tend to involve contusion and some degree of compression and distraction, and either type of injury may result in laceration of the spinal cord by sharp bone fragments or shearing, respectively (Dumont et al., 2001). Even lacerations due to stab injuries in humans regularly involve some degree of contusion/compression due to the impact of the spinal cord with the surrounding vertebrae (Lipschitz and Block, 1962). This is often not the case in preclinical SCI research however, as animal models of SCI typically involve contusion (Allen, 1911; Bresnahan et al., 1987; Noyes, 1987b; Behrmann et al., 1992; Gruner, 1992; Stokes, 1992; Scheff et al., 2003), compression (Rivlin and Tator, 1978; Dolan et al., 1980), distraction (Myklebust et al., 1988; Dabney et al., 2004), partial or complete crush (Plemel et al., 2008), or partial or complete transection (Theriault and Tator, 1994; Chadi et al., 2001; Kwon et al., 2002a; Hendriks et al., 2006; Pettersson et al., 2007) of the spinal cord in near-complete isolation from other mechanisms of injury. Given that most cases of SCI in humans include contusion as a primary mechanism of injury (Bunge et al., 1993; Schwab and Bartholdi, 1996; Dumont et al., 2001; Norenberg et al., 2004), contusive injury models tend to mimic the typical human pathology best, and are therefore most commonly used to study secondary mechanisms of injury and to assess the efficacy of potential treatments in animals (Kwon et al., 2002a; Dietz and Curt, 2006).

Primary injury typically results in two major and immediate pathological events: 1) physical damage to blood vessels in the spinal cord, particularly the microvasculature of the gray matter, causing hemorrhage, edema and impaired microcirculatory tissue perfusion at the site of initial trauma and 2) disruption of neuronal, glial, and endothelial cellular membranes causing the rapid necrotic death of neurons, astrocytes, oligodendrocytes, and endothelial cells at the
lesion epicentre and less severe damage to cells, axons, and myelin in adjacent regions (Hausmann, 2003; Choo et al., 2007; Mothe and Tator, 2012; Mautes et al., 2000). More severe injuries are known to cause more extensive hemorrhage and necrosis (Anderson, 1985; Noyes, 1987a; Boldin et al., 2006), but the initial pattern of neuropathology also varies according to the specific mechanisms of primary injury that occur during SCI. For example, in the case of laceration injuries, the pattern of initial damage is clearly dictated by the trajectory of foreign bodies or bone through the spinal canal, but in all other mechanisms of primary injury it is generally recognized that initial mechanical damage occurs primarily in the central gray matter, where the tissue is less resilient and more vascularized (Wolman, 1965; Kakulas, 1984; Tator and Koyanagi, 1997; Dumont et al., 2001; Norenberg et al., 2004). Thus in most cases, primary injury preferentially affects the capillaries/venules, neurons/axons and glia in the central gray matter.

However, even amongst those primary injury mechanisms that preferentially affect the gray matter there can be substantial variability in the pattern of hemorrhage and cellular membrane compromise. For example, just 5 minutes after injury in the rat, Choo et al. (2007) found that contusion and dislocation injuries both produced substantial hemorrhage in the gray matter at the site of injury, whereas distraction injuries caused little to no noticeable hemorrhage. Furthermore, although contusion and dislocation both resulted in damage to cellular membranes in the gray and white matter at the lesion epicentre, as well as near-immediate tissue necrosis in the gray matter, distraction was not associated with obvious necrosis and both dislocation and distraction were associated with more extensive, diffuse disruption of membranes on neuronal somata and axons both rostral and caudal to injury (Choo et al., 2007). Subsequent work by that
same group demonstrated that the differences in primary injury resulting from those three mechanisms lead to substantial differences in secondary pathology as well (Choo et al., 2008), clearly demonstrating that the mechanism of primary injury plays a key role in determining the pattern and extent of spreading secondary damage; which may explain why the extent of hemorrhage has proven highly predictive of functional outcomes following both experimental and clinical SCI (Noyes, 1987a; Boldin et al., 2006).

1.3.2 Secondary injury

The necrotic death of cells, and the severe axonal and vascular compromise caused by the initial trauma to the spinal cord triggers a cascade of secondary injury mechanisms at the tissue, cellular and molecular levels, which contribute to the degeneration of tissue adjacent to the lesion site that was not damaged by the initial injury itself. Many of these processes are interrelated, arising from and/or contributing to one another in a manner that promotes further tissue loss over time.

1.3.2.1 Secondary injury mechanisms triggered by vascular changes after SCI

Primary injury generally causes a relatively small region of hemorrhage to appear almost immediately at the injury epicentre, but over the next 24 hours, very small regions of hemorrhage (i.e., petechiae) often emerge in adjacent tissue that was spared the initial trauma, and these small hemorrhages coalesce over time into one large hemorrhagic lesion during secondary injury (Simard et al., 2007). This progressive hemorrhagic necrosis is thought to substantially increase the loss of cells, as the volume of hemorrhage typically doubles within 12-18 hours after contusion injuries (Simard et al., 2012). Although a number of destructive processes are likely to contribute to progressive hemorrhagic necrosis during secondary injury, one of the prime culprits
is the non-selective cation channel sulfonylurea receptor 1 (Sur1). Sur1 is not expressed constitutively, but is upregulated *de novo* in both neural and endothelial cells following CNS injury, and this channel has been linked to microvascular (e.g., capillary) fragmentation, leading to vasogenic edema and delayed secondary petechial hemorrhage formation after SCI (Simard et al., 2012).

As the term hemorrhagic necrosis implies, hemorrhage is well associated with the necrotic death of neural cells. Although there are many potentially neurotoxic substances that may be present in blood, the majority of necrosis due to hemorrhage appears to result from hemoglobin neurotoxicity (Regan et al., 2008). The degradation of hemoglobin generates iron, which accumulates in tissue following hemorrhage and can persist for months thereafter (Hua et al., 2006). As a catalytic metal ion, iron serves as a substrate for free radical chain reactions, particularly during lipid peroxidation (Mautes et al., 2000), and thus causes oxidative damage to cells (see below), leading to necrosis in cells that accumulate too much damage to survive. Neurons are particularly vulnerable to hemoglobin toxicity, primarily due to their limited capacity to sequester and detoxify iron (Regan et al., 2008).

In addition to hemorrhage, SCI is also associated with widespread edema and ischemia (Dumont et al., 2001). Vasogenic edema results from a breakdown of the blood-spinal cord barrier (BSCB), which occurs due to the damage/loss of endothelial cells and astrocytes that maintain the tight endothelial junctions of the BSCB (Saadoun and Papadopoulos, 2010; Bartanusz et al., 2011). Ischemia is thought to result from a combination of local vascular damage, local responses to that damage (i.e., vasospasm, thrombosis, and a loss of autoregulatory homeostasis), and systemic hypoperfusion that occurs due to injury-induced
neurogenic shock; the latter of which is a common cardiovascular response to severe SCI, characterized by bradycardia, hypotension, and decreased cardiac output and peripheral resistance (Dumont et al., 2001). As secondary injury progresses, vascular damage and BSCB breakdown spreads, as numerous vasoactive and/or endothelial destructive compounds (e.g., endothelin-1, excitatory neurotransmitters, reactive oxygen species, bradykinins, and histamines) are released into the local environment due to the compromise of neural and endothelial cellular membranes, the activation of adjacent endothelial and glial cells (particularly astrocytes), and the arrival of blood-borne inflammatory cells (Noble and Wrathall, 1989; Tator and Fehlings, 1991; McKenzie et al., 1995; Popovich et al., 1996b; Schnell et al., 1999; Bartanusz et al., 2011). Thus, the breakdown of the BSCB spreads to spared segments both rostral and caudal to injury, peaking within days of injury and gradually declining over the subsequent weeks (Noble and Wrathall, 1989; Popovich et al., 1996b; Schnell et al., 1999; Bilgen et al., 2001), but remaining elevated even 8 weeks after SCI in rats (Cohen et al., 2009). As a result of the combined effect of those events, spinal cord tissue (particularly at the lesion epicentre and in adjacent regions) is subjected to ischemia, edema, disturbed ionic homeostasis, and exposure to a variety of cells and proteins normally excluded from the CNS by the BSCB (Griffiths and Miller, 1974; Beggs and Waggener, 1975, 1976; Lemke et al., 1987; Noble and Wrathall, 1988; Tator and Fehlings, 1991; Popovich et al., 1996b).

Vasogenic edema caused by BSCB dysfunction causes swelling of the spinal cord that can lead to compression against the surrounding dura, resulting in high intraparenchymal pressure that contributes to ongoing ischemia (Saadoun et al., 2008; Saadoun and Papadopoulos, 2010). Damage to the vasculature also provides a route for the invasion of the spinal cord by
blood-borne inflammatory cells (see below) and the breakdown of the BSCB releases serum into the spinal cord parenchyma that likely contributes to the activation of resident microglia (Ransohoff and Perry, 2009; Takigawa et al., 2010), even in regions relatively removed from the lesion site. The necrotic death of cells due to severe membrane disruption, and widespread hemorrhage, edema, and ischemia serves to perpetuate secondary damage, due to the uncontrolled release of ions, excitatory neurotransmitters, and reactive oxygen species into the extracellular space. However, of all the vascular events induced by SCI, ischemia may contribute the most to secondary injury, as it triggers the widest array of secondary cytotoxic events that contribute directly to the progressive loss of neuronal and glial cells over time (Tator and Fehlings, 1991; Pantoni et al., 1996; Mautes et al., 2000; Dumont et al., 2001; Hausmann, 2003; Szydlowska and Tymianski, 2010).

Ischemia results in a lack of sufficient oxygen (hypoxia) and glucose to support cellular metabolism, leading to a reduction in the synthesis of adenosine triphosphate (ATP) and the rapid depletion of cellular energy (i.e., ATP stores) that is required to maintain the ionic gradients necessary for cellular function and homeostasis in the CNS (Saikumar et al., 1998; Harris and Attwell, 2012). As a result of this energy failure, ischemia results in cellular edema, chronic membrane depolarization, the release of excitatory neurotransmitters (e.g., glutamate), and reduced neurotransmitter reuptake from the extracellular space (Liang et al., 2007; Szydlowska and Tymianski, 2010). Cellular edema almost inevitably leads to oncotic cell death, which is a form of necrotic cell death due to cytotoxic swelling, but it also contributes to vasogenic edema and may therefore serve to further exacerbate ischemia following SCI (Liang et al., 2007; Saadoun and Papadopoulos, 2010). Although all CNS cell types swell during ischemia,
Astrocytes appear to be particularly vulnerable to this phenomenon, due to the presence of aquaporin-4 water channels on the astrocytic endfeet that directly contact endothelial cells of the BSCB (Swanson et al., 2004; Saadoun and Papadopoulos, 2010).

The release of excitatory amino acids following SCI occurs due to ischemia, but also due to necrotic cell death and SCI-induced astrocyte dysfunction (see below), and in combination those events cause extracellular glutamate to reach excitotoxic levels rapidly following SCI (Panter et al., 1990; Liu et al., 1991; Wrathall et al., 1996). The accumulation of glutamate in the extracellular space activates glutamate receptors (e.g., N-Methyl-D-aspartate [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] and kainate receptors), which induces the release of even more glutamate into the extracellular space (Zhou et al., 2013), thus contributing to excessive glutamate receptor activation; the net effect of which is a rise in intracellular calcium (Ca^{2+}) (Choi, 1988; Matute et al., 2007). In addition to glutamate receptors, the intracellular accumulation of Ca^{2+} may also occur under ischemic conditions, due to the dysfunctional activation of a variety of other channels and transporters (e.g., TRPM7, NCX, P2X_7) and/or the release of calcium from internal stores in the endoplasmic reticulum (Szydlowska and Tymianski, 2010; Arbeloa et al., 2012). The excessive intracellular accumulation of Ca^{2+} is associated with a wide range of harmful downstream events, including: the over-activation of various proteases, lipases, phosphatases, and endonucleases; further disruption of ion transporters; increased production of reactive oxygen species; endoplasmic reticulum dysfunction; and mitochondrial dysfunction (Kroemer et al., 1998; Dumont et al., 2001; Szydlowska and Tymianski, 2010). Those processes culminate in a variety of negative consequences for the cell, including the persistence of Ca^{2+} elevation, acidosis, cellular edema,
reduced ATP synthesis, increased oxidative and electrophilic stress, DNA fragmentation, cytoskeletal breakdown, and damage to cellular and/or mitochondrial membranes (Lewen et al., 2000; Higuchi et al., 2005; Szydlowska and Tymianski, 2010). In regions of severe/prolonged ischemia, such as the lesion epicentre, these processes commonly lead to necrotic cell death, but in regions subjected to sub-lethal ischemia, apoptotic cell death (which requires energy) is more likely, and is often triggered by the reoxygenation when tissue perfusion improves (Saikumar et al., 1998; Dumont et al., 2001; Szydlowska and Tymianski, 2010). Mitochondrial damage caused by elevated intracellular Ca\(^{2+}\) is thought to play a key role in apoptosis due to ischemia/reperfusion, as this leads to the release of cytochrome c, which acts in concert with apoptosis activating factor-1 to activate caspase-9 to induce caspase-3 and caspase-6, leading to programmed cell death (Budd et al., 2000; Eldadah and Faden, 2000; Kuida, 2000).

Oxidative stress resulting from the elevated production of reactive oxygen species (ROS) during ischemia, is elevated even further during reperfusion, and is thought to play a particularly important role in secondary damage following SCI (Demopoulos et al., 1982; Hall and Braughler, 1982; Anderson et al., 1985; Braughler et al., 1987; Hall et al., 1992; Hamann et al., 2008; Yune et al., 2008; Hamann and Shi, 2009), as this process leads to the oxidation of proteins, lipids, and nucleic acids causing macromolecular damage to cellular structures, including the cell membrane and mitochondria, the latter of which may result in permanently decreased ATP synthesis and increased ROS production (Schmidley, 1990; Barja, 2004; Jia et al., 2012). The CNS is particularly susceptible to oxidative stress due to the high concentration of polyunsaturated fatty acids that are vulnerable to lipid peroxidation in neuronal and glial cell membranes, the high rate of oxidative metabolic activity, the related production of reactive
oxygen and nitrogen metabolites, and the relatively low concentration of antioxidants (Hall et al., 1992; Storey, 1996; Vaziri et al., 2004; Logan et al., 2005; Hamann et al., 2008). Widespread lipid peroxidation is known to occur after SCI, and contributes substantially to membrane damage (Jia et al., 2012), but also produces non-radical oxidants such as the conjugated aldehyde, acrolein, which is thought to be one of the main culprits responsible for membrane damage, mitochondrial dysfunction and myelin disruption (Hamann et al., 2008; Hamann and Shi, 2009; Shi et al., 2011). The half-life of acrolein is much longer than that of conventional ROS, so it can diffuse out from injured tissue into adjacent spared tissue, propagating secondary injury more readily (Shi et al., 2011).

1.3.2.2 Secondary damage mediated by inflammatory cells following SCI

Neutrophils are the first blood-borne inflammatory cells to arrive in large numbers after SCI, as within hours of injury these cells begin to accumulate in the vascular endothelium and the parenchyma adjacent to regions of hemorrhagic necrosis, peaking in number at 12-24 hours in the rat and 1-3 days in humans, and disappearing from the injured cord altogether within about 5-10 days in both of those species (Taoka et al., 1997; Carlson et al., 1998; Fleming et al., 2006; Beck et al., 2010). Although neutrophils help to sterilize the injury site via the release of oxidative and proteolytic enzymes, excessive release of those substances due to the robust neutrophil response following SCI is thought to cause damage to uninjured (‘bystander’) cells in adjacent spared tissue (Taoka et al., 1997), and the release of the protease elastase may play a particularly important role in facilitating continued secondary damage by injuring endothelial cells to further increase vascular permeability (Harlan, 1987; Zimmerman and Granger, 1990; Carlos and Harlan, 1994; Taoka et al., 1998).
Resident microglia in the CNS are known to respond to local tissue damage within seconds of injury (Davalos et al., 2005; Nimmerjahn et al., 2005; Hines et al., 2009), at least in part due to the activation of P2Y$_{12}$ purinergic receptors by nucleotides (e.g., ATP) released into the extracellular space by damaged cells and reactive astrocytes (Davalos et al., 2005; Haynes et al., 2006), but perhaps also in response to serum components and/or extracellular glutamate released as a result of damage to endothelial and/or neural cells (Ransohoff and Perry, 2009). In response to injury, activated microglia proliferate, upregulate the expression of cytokines (e.g., interleukin-1 [IL-1] and tumour necrosis factor alpha [TNFα]) and chemokines (e.g., leucotrienes) and eicosanoids (e.g., prostaglandins), and these cells often take on a phagocytic phenotype that is indistinguishable from hematogenous macrophages (Hausmann, 2003; David and Kroner, 2011). The number of activated microglia peaks within the lesion site by 1 week post-injury and in the white matter by 3 weeks later in the rat (Popovich et al., 1997). At the same time, hematogenous macrophages begin to accumulate at the lesion site in the injured spinal cord, peaking around 5-7 days post-injury (Popovich et al., 1997; Carlson et al., 1998; Fleming et al., 2006).

Unlike neutrophils, activated microglia and phagocytic macrophages (from resident microglia or hematogenous monocytes) persist in the injured spinal cord for weeks to months after SCI in both the rat and human (Popovich et al., 1997; Carlson et al., 1998; Fleming et al., 2006). Although these cells play important roles in the clearance of debris and tissue repair, they are also known to cause bystander damage in tissue adjacent to the lesion site, via the release of a variety of neurotoxic compounds including proteolytic enzymes, free radicals (e.g., nitric oxide [NO]), and pro-inflammatory cytokines (e.g., IL-1 and TNFα) (Chao et al., 1992; Hausmann,
Following SCI, the macrophages found in the spinal cord predominantly display a pro-inflammatory or ‘M1’ phenotype (Mosser and Edwards, 2008; Kigerl et al., 2009). In tissues outside of the CNS, this pro-inflammatory (i.e., “M1”) macrophage response eventually declines as M1 macrophages are replaced by anti-inflammatory or “M2” macrophages (Mosser and Edwards, 2008; Laskin, 2009). However, this transition does not occur following SCI, where pro-inflammatory M1 macrophages continue to dominate the lesion indefinitely, thus contributing substantially to secondary tissue damage following SCI (Kigerl et al., 2009).

T-lymphocytes also invade the injury site during the first week after SCI, predominantly within the lesion epicentre, and although their numbers remain elevated for 6-10 weeks after injury, the number of these cells remains relatively low compared to other inflammatory cells recruited after SCI (Popovich et al., 1997; Schnell et al., 1999; Sroga et al., 2003; Fleming et al., 2006). Lymphocytes can lyse oligodendrocytes directly (Antel et al., 1994) and/or induce apoptosis by releasing molecules that activate cellular death receptors (Almad et al., 2011), and although the deliberate addition of CNS-reactive T-cells has been shown to contribute to damage and functional deficits following SCI (Popovich et al., 1996a; Jones et al., 2002), the extent to which those cells contribute to naturally occurring secondary damage after SCI remains a matter of some debate (Popovich and Jones, 2003; Crutch et al., 2006).

1.3.2.3 Cell death and degeneration following SCI

Whereas primary injury results in the rapid necrotic death of many neurons, astrocytes and oligodendrocytes after SCI, secondary injury contributes even further to cell losses starting immediately after primary injury and continuing for weeks thereafter. For example, secondary
damage appears as early as 24 hours after contusion injury, as the spared/intact ventromedial white matter near the injury site shows a significant loss of axons, the development of severe myelin pathology, and a 50% reduction in the number of oligodendrocytes and astrocytes (Rosenberg and Wrathall, 1997; Grossman et al., 2001), but the apoptotic death of oligodendrocytes in the spared white matter adjacent to injury continues for at least 3 weeks following contusion in rats, and is thought to contribute substantially to the demyelination of intact axons that survived the initial trauma (Blight, 1983, 1985; Shuman et al., 1997; Totoiu and Keirstead, 2005; Almad et al., 2011). Apoptosis occurs in both gray and white matter, and appears to be most prominent in oligodendrocytes, neurons, and microglia, but can also effect astrocytes after severe SCI (Crowe et al., 1997; Liu et al., 1997; Lou et al., 1998; Yong et al., 1998; Casha et al., 2001). Following SCI apoptosis is thought to occur via multiple pathways, including extrinsic (receptor-mediated) apoptosis induced by activation of death receptors (e.g., Fas and p75) (Casha et al., 2001) and/or the production of inducible nitric oxide synthase (iNOS) by macrophages (Satake et al., 2000), and intrinsic (receptor-independent) apoptosis induced by mitochondrial damage (Eldadah and Faden, 2000) or direct activation of the caspase-3 proenzyme (Citron et al., 2000). The contribution of apoptosis to secondary damage is perhaps best demonstrated by evidence that the inhibition of caspases (mediators of apoptosis) reduces lesion size and improves motor performance after SCI in mice (Li et al., 2000).

In addition to widespread necrosis and apoptosis, autophagic cell death has recently been reported in neurons, astrocytes, but especially oligodendrocytes for at least 21 days following SCI in mice (Kanno et al., 2009; Kanno et al., 2011) and has been implicated in the loss of neurons following contusion injury in rats (Chen et al., 2012a). Under normal or mild
pathological conditions, autophagy is thought to be a protective cellular response, as it allows for the removal of aggregated proteins and damaged organelles, including mitochondria, thereby promoting the health and longevity of cells (Mao and Reddy, 2010; Xu and Zhang, 2011). However, excessive autophagy can be triggered by severe cellular insults, such as those associated with SCI, and may actively contribute to axonal degeneration, and cellular atrophy and death (Cherra and Chu, 2008; Xu and Zhang, 2011).

Besides contributing to the loss of cells due to secondary damage, apoptosis is known to continue for weeks and even months after SCI in adult rodents, monkeys, and even humans in axon tracts undergoing Wallerian degeneration (Katoh et al., 1996; Li et al., 1996; Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997; Emery et al., 1998; Lou et al., 1998; Yong et al., 1998; Abe et al., 1999; Li et al., 1999; Casha et al., 2001; Warden et al., 2001). Wallerian degeneration is a normal response to injuries that involve axonal transection in both the PNS and the CNS, and refers specifically to the disintegration of distal axon segments that have been cut off from their neuronal somata and the degradation of associated myelin sheaths (Vargas and Barres, 2007; Gaudet et al., 2011). In the PNS, Wallerian degeneration only takes 1-2 weeks to complete, and is thought to play an essential role in remodelling the environment of the distal nerve segment into one that supports the successful regeneration of injured axons (Griffin et al., 1992; George and Griffin, 1994; Gaudet et al., 2011), however, in the adult mammalian CNS, the rate of Wallerian degeneration is much slower, taking months to years to reach completion after SCI (Bignami and Ralston, 1969; Miklossy and Van der Loos, 1987; Perry et al., 1987; Miklossy and Van der Loos, 1991; George and Griffin, 1994; Becerra et al., 1995; Buss et al., 2004). As a result, the degeneration of axons and myelin and the loss of oligodendrocytes continue for a very
long time in injured axon tracts rostral and caudal to the injury site after SCI, but this process is distinct from secondary injury as the loss of axons and myelin from tracts that have already been severed by SCI does not appear to result in any additional meaningful loss of function (Vargas and Barres, 2007).

1.3.3 The response of neural cells to SCI

In addition to cell death, the responses of local stem/progenitor cells, neurons, oligodendrocytes, and astrocytes also play a role in determining the extent of secondary damage and/or spontaneous recovery following SCI.

1.3.3.1 Spinal cord stem/progenitor cells

The existence of multiple populations of neural stem/progenitor cells (NSPCs) or oligodendrocyte progenitor cells (OPCs) in the adult mammalian spinal cord is well established, with proposed niches in the ependymal, sub-pial, and intraparenchymal regions (Johansson et al., 1999; Namiki and Tator, 1999; Horner et al., 2000; Yamamoto et al., 2001a; Martens et al., 2002; Barnabe-Heider et al., 2010; Petit et al., 2011). Many of these cells are known to proliferate extensively and rapidly in response to SCI (Johansson et al., 1999; Namiki and Tator, 1999; Yamamoto et al., 2001a; Mothe and Tator, 2005; Zai and Wrathall, 2005; Barnabe-Heider et al., 2010), beginning as early as 24 hours after injury (Horky et al., 2006), generally peaking within 1 week post-injury (wpi), and subsiding to insignificant levels by 4 wpi (McTigue et al., 2001; Zai and Wrathall, 2005; Zai et al., 2005). NSPCs can be isolated and differentiated into neurons, astrocytes, and oligodendrocytes under appropriate culture conditions (Weiss et al., 1996; Shihabuddin et al., 1997; Yamamoto et al., 2001a; Kulbatski et al., 2007; Mothe et al.,
and the transplantation of human NSPCs from spinal cord ependyma after SCI in the rat has been shown to produce neurons as well as glia \textit{in vivo} (Mothe et al., 2011).

However, despite all of the evidence that NSPCs in the adult mammalian spinal cord are capable of generating neurons, spontaneous neurogenesis largely fails to occur in the adult mammalian spinal cord under normal conditions (Horner et al., 2000; Horky et al., 2006) or following injury (Yamamoto et al., 2001b; Mothe and Tator, 2005; Zai and Wrathall, 2005; Horky et al., 2006). Instead spinal progenitor cells preferentially generate glia, and thus replace lost astrocytes and/or oligodendrocytes, but not neurons following SCI (Johansson et al., 1999; Horner et al., 2000; Martens et al., 2002; Mothe and Tator, 2005; Zai and Wrathall, 2005; Horky et al., 2006; Yang et al., 2006; Vessal et al., 2007; Meletis et al., 2008). Thus, despite the fact that approximately 50\% of the oligodendrocytes and astrocytes in the spared white matter die within 24 hours after contusion injury (Grossman et al., 2001), glial densities generally return to normal levels in the spared tissue rim between 2-6 weeks post-injury (Wrathall et al., 1998; Frei et al., 2000; Rosenberg et al., 2005; Tripathi and McTigue, 2007), and this occurs concurrent with ongoing apoptosis in those regions (Crowe et al., 1997; Liu et al., 1997; Yong et al., 1998; Li et al., 1999; Warden et al., 2001).

\subsection*{1.3.3.2 Glial scar formation and astrocyte contributions to secondary injury}

Although many astrocytes die during primary and secondary injury (Jaeger and Blight, 1997; Yong et al., 1998; Grossman et al., 2001), surviving astrocytes in the vicinity of the lesion site respond to SCI by undergoing reactive astrogliosis. That process involves proliferation followed by hypertrophy and increased production of glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs) by astrocytes at the site of injury (Eng and Ghirnikar,
1994; Fitch and Silver, 1997; Popovich et al., 1997; Stichel and Muller, 1998; Fawcett and Asher, 1999; Schnell et al., 1999). The number of reactive astrocytes in the lesion site increases over time and these cells surround and enclose the injury site, forming a physical barrier commonly referred to as the glial scar, which walls off areas of necrosis from the surrounding spared parenchyma (Faulkner et al., 2004; Okada et al., 2006; Wanner et al., 2013) and acts, in combination with increased CSPG production, to inhibit axonal regeneration through the site of injury (Reier et al., 1983; Davies et al., 1997; Popovich et al., 1997; Davies and Silver, 1998; Davies et al., 1999; Fawcett and Asher, 1999; McKeon et al., 1999; Hermanns et al., 2001; Bradbury et al., 2002; Silver and Miller, 2004). In addition, newly generated astrocytes that arise from spinal cord stem/progenitor cells also contribute to glial scar formation (Johansson et al., 1999; Mothe and Tator, 2005; Meletis et al., 2008; Barnabe-Heider et al., 2010), and some of those cells appear to generate a transient population of phagocytic astrocytes early after hemisection SCI; at least in mice (Sellers et al., 2009). The formation of the glial scar is thought to limit the spread of hematogenous inflammatory cells and help restore the BSCB, and therefore astrocytes are generally thought to play a protective role following SCI as they limit the spread of damage into adjacent uninjured tissue (Fitch and Silver, 1997; Stichel and Muller, 1998; McGraw et al., 2001; Okada et al., 2006; Sofroniew, 2009). However, the astrocyte response to SCI appears to come at the expense of axonal regeneration, as the end result of reactive astrogliosis is a lesion site that is much less accessible to regenerating CNS axons (Silver and Miller, 2004; Busch and Silver, 2007).

In addition to the formation of the glial scar, and in spite of numerous potentially protective functions served by these cells (e.g., uptake of excitatory glutamate), astrocytes have
also been implicated in the exacerbation of secondary injury, particularly due to their role in modulating injury-induced inflammation (Swanson et al., 2004; Farina et al., 2007). Following injury astrocytes release ATP to trigger microglial activation (Davalos et al., 2005) and produce a variety of chemokines (e.g., CXCL10 and CCL2) and cytokines (e.g., TNFα, IL-1 and IL-6) that further propagate the inflammatory response by promoting inflammatory cell infiltration and proliferation (Swanson et al., 2004; Brambilla et al., 2005; Brambilla et al., 2009). Indeed, transgenic inhibition of the transcription factor nuclear factor kappa B (NF-κB; a key regulator of inflammation and secondary injury processes after SCI) specifically in astrocytes is associated with reduced inflammation, and enhanced neuroprotection and functional recovery after contusive SCI in mice (Brambilla et al., 2005). Astrocytes are also implicated in the development of both vasogenic and cytotoxic edema, because they secrete the angiogenic factor vascular endothelial growth factor A (VEGF-A) and matrix metalloproteases (e.g., MMP2), which are both known to contribute directly to BSCB disruption (Swanson et al., 2004; Argaw et al., 2009), and they express aquaporin-4 which is elevated after SCI, making astrocytes highly susceptible to cytotoxic edema, which can spread to adjacent cells via gap junctions and may lead to increased vasogenic edema (Swanson et al., 2004; Liang et al., 2007; Saadoun and Papadopoulos, 2010). In addition, astrocytes show enhanced NO production in the presence of elevated transforming growth factor β1 (TGF-β1) (Hamby et al., 2006), and they release glutamate rather than taking it up in the presence of elevated TNFα (Bezzi et al., 2001). Given that both astrocytes and microglia produce TGF-β1 and TNFα following injury (John et al., 2003), the release of NO and glutamate from astrocytes likely contributes to ongoing excitotoxicity and free radical and lipid peroxidation damage after SCI.
1.3.3.3 The oligodendrocyte response to SCI: Demyelination vs. Remyelination

In contrast to the resilient and multifaceted (although somewhat detrimental) response to injury displayed by astrocytes, the primary responses of oligodendrocytes following injury appear to be necrosis, apoptosis and quiescence (Ludwin, 1990; Crowe et al., 1997; Liu et al., 1997; Li et al., 1999; Casha et al., 2001; Almad et al., 2011). Oligodendrocytes are particularly susceptible to oxidative stress and glutamate excitotoxicity during ischemia (Merrill et al., 1993; Oka et al., 1993; Noble et al., 1994; Husain and Juurlink, 1995; Pantoni et al., 1996; McDonald et al., 1998b; McDonald et al., 1998a; Li and Stys, 2000; Matute et al., 2007), and also appear to be vulnerable to elevated extracellular ATP (Wang et al., 2004; Matute et al., 2007; Huang et al., 2012) as well as elevated levels of proinflammatory cytokines (e.g., TNFα and IL-1β) (Vartanian et al., 1995; Curatolo et al., 1997; Sherwin and Fern, 2005; Li et al., 2008; Steelman and Li, 2011). In addition, oligodendrocytes are known to express cell surface ‘death receptors’ including Fas and p75, the activation of which appear to be involved in delayed apoptosis in white matter tracts following SCI (Casha et al., 2001; Beattie et al., 2002), and although those receptors are also expressed on astrocytes, the latter are largely resistant to death receptor-induced apoptosis (Song et al., 2006). As a result of these vulnerabilities, large numbers of oligodendrocytes are lost following SCI, with 93% of those cells dying within 7 days post-injury at the lesion epicentre (McTigue et al., 2001), and ongoing apoptosis in the adjacent degenerating white matter tracts for 3-6 weeks after contusion injuries in rats and monkeys (Crowe et al., 1997; Shuman et al., 1997) and at least 60 days after SCI in humans (Emery et al., 1998). Indeed, the majority of apoptosis of neural cells following SCI appears to involve oligodendrocytes rather than neurons, particularly during Wallerian degeneration of injured axon tracts in the white matter rostral and caudal to injury (Crowe et al., 1997; Liu et al., 1997; Shuman et al., 1997; Almad et al., 2011).
Although primary and secondary injury both cause direct damage to myelin sheaths, the loss of oligodendrocytes due to prolonged apoptosis in spared white matter tracts is thought to largely contribute to the demyelination of intact axons adjacent to the lesion site following SCI (Crowe et al., 1997; Shuman et al., 1997; Casha et al., 2001); particularly considering that each oligodendrocyte myelinates segments on 30-80 different axons (Chong et al., 2012). Demyelination is a prominent feature after SCI in both animals (Gledhill et al., 1973a; Bresnahan et al., 1976; Blight, 1983, 1985; Totoiu and Keirstead, 2005; Smith and Jeffery, 2006; Siegenthaler et al., 2007; Lasiene et al., 2008; James et al., 2011; Powers et al., 2012), and humans (Bunge et al., 1993; Kakulas, 1999; Norenberg et al., 2004; Guest et al., 2005). However, spontaneous remyelination is also a consistent finding after SCI, typically beginning around 2-3 weeks post-injury (Bunge et al., 1961; McDonald and Ohlrich, 1971; Gledhill et al., 1973a; McDonald, 1975; Harrison and McDonald, 1977; Kakulas, 1999; Totoiu and Keirstead, 2005; Smith and Jeffery, 2006; Lasiene et al., 2008; Powers et al., 2012), and is associated with the restoration of saltatory conduction with normal or near-normal action potential conduction properties (Smith et al., 1979; James et al., 2011). Remyelinated axons have traditionally been identified by abnormally thin or short myelin sheaths (i.e., larger g-ratios [axon diameter / total fibre thickness (including myelin)] and shorter internodal lengths), largely based on electron microscopy (EM) work from the 1970s and 1980s, describing presumptive remyelinated CNS fibres a few weeks after demyelination (Bunge et al., 1961; Gledhill and McDonald, 1977). However, recent work using an inducible membrane-bound reporter to label glial progenitor cells in the injured spinal cord of transgenic mice allowed for more definitive identification of regenerated myelin after SCI, and revealed that newly generated oligodendrocyte myelin sheaths thicken over time, approaching uninjured values (based on g-ratio) within 6 months post-injury,
but remaining significantly thinner than newly generated SC myelin sheaths (Powers et al., 2013). Thus, newly formed oligodendrocyte myelin sheaths do not remain thinner than the myelin sheaths formed during natural development (Powers et al., 2013).

Despite ongoing remyelination, the presence of denuded axons in the spinal cords of chronically injured animals, and to lesser extent in human tissue, has long been viewed as evidence that endogenous remyelination is insufficient following SCI, and thus represents a therapeutic target even in chronic SCI (Gledhill et al., 1973b; Blakemore, 1974; Gledhill and McDonald, 1977; Blight, 1983; Bunge et al., 1993; Guest et al., 2005; Totoiu and Keirstead, 2005). However, more recent evidence indicates that the techniques used to estimate the number of demyelinated fibres in earlier work likely included demyelinated segments of degenerating/severed axons, thereby overestimating the extent of demyelination that is functionally relevant. Indeed, all intact/functional axons are remyelinated by 3 months after SCI in mice and rats, which suggests that only injured / degenerating / dysfunctional axons remain demyelinated in the chronic injury setting (Lasiene et al., 2008; Powers et al., 2012). Thus, although demyelination may persist in the chronic SCI setting, it appears to be a target of limited utility, as the only axons that need remyelinating are those that are damaged or dysfunctional (Powers et al., 2012). Given the injured axons are likely to degenerate over time, this may explain why only limited demyelination has been observed in most human tissue from patients with chronic SCI examined years/decades after injury, except in conditions involving some degree of ongoing cord compression (Bunge et al., 1993; Gensert and Goldman, 1997; Kakulas, 1999; Norenberg et al., 2004; Guest et al., 2005).
Mature oligodendrocytes are post-mitotic, and thus unable to divide to replace the cells that are lost following SCI (Keirstead and Blakemore, 1997), but new myelinating oligodendrocytes are generated by one of the resident spinal cord progenitor populations, known as oligodendrocyte progenitor cells (OPCs) or polydendrocytes. OPCs are typically identified by the expression of nerve/glial antigen 2 (NG2) or platelet-derived growth factor receptor alpha (PDGFRα) (Nishiyama et al., 2009; Trotter et al., 2010). These cells are present throughout the gray and white matter in the adult CNS, and respond to demyelination by proliferating, migrating to areas of demyelination, and remyelinating denuded axons (Wolswijk and Noble, 1989; Carroll et al., 1998; Blakemore and Keirstead, 1999; Keirstead and Blakemore, 1999; Watanabe et al., 2002; Lytle et al., 2009). In addition to newly generated oligodendrocytes, endogenous SCs are also well known to play a role in remyelination in the injured CNS, particularly following contusion or in demyelinating injury/disease states (Bunge et al., 1993; McTigue et al., 2001; Guest et al., 2005; Totoiu and Keirstead, 2005). While traditionally those cells were assumed to have migrated into the injured spinal cord from the periphery, recent evidence suggests that the majority of the endogenous myelinating SCs found in the CNS after toxin-induced focal demyelination actually arise from local CNS precursors, rather than peripheral origins (Zawadzka et al., 2010).

Although spontaneous remyelination by OPCs and SCs ensures that the vast majority of intact axons do not remain permanently denuded after SCI, remyelination by endogenous cells is delayed following SCI, so demyelination is quite prevalent during the first couple of weeks following SCI (Gledhill et al., 1973a; Harrison and McDonald, 1977; Blight, 1985), and appears to affect a large number of axons in spared white matter tracts, given that 53% of spared
rubrospinal tract axons showed signs of remyelination following contusion injury in the rat (Powers et al., 2012). Demyelination has long been known to result in the impairment of rapid axonal conduction (Bostock and Sears, 1976; Bostock et al., 1978), but more recent evidence indicates that myelinating glia are also required for the maintenance of normal axon transport and long-term survival in long projecting axons (Nave and Trapp, 2008; Nave, 2010a, b). Furthermore, recent work has demonstrated that demyelination and/or the depletion of oligodendrocytes are both associated with increased axonal degeneration, and that efficient remyelination is protective against demyelination-associated axonal damage and degeneration (Irvine and Blakemore, 2008; Pohl et al., 2011). Even less severe alterations of oligodendrocyte function, such as the deletion of certain myelin proteins (e.g., proteolipid protein [PLP]; 2´, 3´-cyclic nucleotide 3´-phosphodiesterase [CNPase]; myelin associated glycoprotein [MAG]) (Griffiths et al., 1998; Sheikh et al., 1999; Lappe-Siefke et al., 2003; Pan et al., 2005; Petzold, 2005; Nguyen et al., 2009) or the disruption of oligodendrocyte peroxisomes (Kassmann et al., 2007) have been found to lead to axonal degeneration, indicating the critical nature of oligodendrocyte support for axons under normal conditions. In the pathological environment of the injured spinal cord the dependence of axons on oligodendrocyte-derived support is probably even greater, as bare axons are likely to be more vulnerable to toxic insults and to require increased metabolic support (Redford et al., 1997; Dutta and Trapp, 2007; Nave and Trapp, 2008; Nave, 2010a, b). Thus, although demyelination appears to be a transient phenomenon after SCI, even temporary demyelination increases the vulnerability of axons to secondary damage, and may therefore contribute to the early loss of intact axons in the spared white matter following injury (Lasiene et al., 2008; Almad et al., 2011; Powers et al., 2012). As such, facilitating remyelination in the acute and sub-acute setting after SCI remains a viable
therapeutic target, as earlier remyelination may prevent the loss of some axons due to secondary injury and thereby preserve some neurological function. This may explain why increased remyelination following NSPC transplantation in sub-acute SCI is often correlated with improved functional recovery in animal models (Cao et al., 2005; Cummings et al., 2005; Hofstetter et al., 2005; Keirstead et al., 2005; Lee et al., 2005; Mitsui et al., 2005; Karimi-Abdolrezaee et al., 2006).

In addition to facilitating axonal degeneration, demyelination results in the accumulation of myelin debris for many months following SCI, and in light of the protracted nature of Wallerian degeneration in the CNS (Vargas and Barres, 2007), that debris tends to linger in degenerating axon tracts for a very long time, persisting for months and even years after SCI (Perry et al., 1987; George and Griffin, 1994; Becerra et al., 1995; Buss et al., 2004). Myelin debris is known to inhibit the differentiation of OPCs, which may partly explain the lag in spontaneous remyelination following SCI (Kotter et al., 2006; Baer et al., 2009; Plemel et al., 2013), as the rate of remyelination in the CNS correlates with the rate of myelin clearance (Shields et al., 1999; Zhao et al., 2006; Ruckh et al., 2012). In addition, myelin contains a variety of proteins that are known to inhibit axonal regeneration, including: Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), MAG (McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002), repulsive guidance molecule (RGMa) (Hata et al., 2006), ephrinB3 (Benson et al., 2005), semaphorin4D (Moreau-Fauvarque et al., 2003), and netrin-1 (Löw et al., 2008). As such, myelin debris makes the environment of the injured CNS less favourable to axonal growth and thus contributes to the
failure of axonal regeneration following CNS injury (Filbin, 2003; Schwab, 2004; Liu et al., 2006; Yiu and He, 2006; Cafferty et al., 2010).

**1.3.3.4 Neuronal and axonal responses to SCI: The failure of CNS regeneration**

The neuronal response to SCI is largely similar to that of oligodendrocytes, as primary injury generally results in widespread necrosis of neurons, and both apoptosis and autophagy contribute to more extensive cell losses during secondary injury (Crowe et al., 1997; Liu et al., 1997; Lou et al., 1998; Yong et al., 1998; Kanno et al., 2009; Kanno et al., 2011; Chen et al., 2012a). Unlike lost oligodendrocytes and astrocytes, NSPCs fail to spontaneously replace lost neurons following SCI (see above). In addition to the death of neurons, SCI is well associated with widespread axonal injury and degeneration. Primary injury causes the physical disruption of axons that pass through the lesion site, whereas secondary injury contributes to damage to axons adjacent to the site of the initial trauma, leading to the transection of axons in the ascending and descending axon tracts that pass through the level of injury in the white matter adjacent to injury (Schwab and Bartholdi, 1996). Although the response of CNS axons to transection can vary substantially (Hawthorne et al., 2011), most axons show a similar pattern of behavior following SCI, wherein the distal segments of severed axons undergo protracted Wallerian degeneration and the proximal segments persist near the lesion site, displaying dystrophic, swollen endbulbs that lack filopodia and likely provide no meaningful function (Coleman and Perry, 2002; Guízar-Sahagún et al., 2004; Tom et al., 2004). While some populations of axons (e.g., serotonergic fibres) are known to persist at the lesion edge and show considerable spontaneous sprouting, the proximal segments of transected cortical axons typically retract from the lesion site (Houle and Jin, 2001; Hawthorne et al., 2011).
Thus, in contrast to the outcome of PNS injury, spontaneous axonal regeneration fails following injury to the adult mammalian CNS. The failure of CNS regeneration is thought to arise from a number of factors, including: 1) the lack of an adequate intrinsic regenerative response in mature CNS neurons (Cai et al., 2001; Bulsara et al., 2002; Harel and Strittmatter, 2006; Szpara et al., 2007); 2) the lack of growth permissive substrates at the lesion site, including the presence of gaps and/or large cystic cavities (McDonald, 1999); 3) the formation of the inhibitory glial scar (Silver and Miller, 2004; Yiu and He, 2006; Busch and Silver, 2007); 4) the persistence of inhibitory myelin proteins, including Nogo, MAG, and OMgp (McGee and Strittmatter, 2003; Liu et al., 2006; Cafferty et al., 2010); 5) the lack of adequate trophic support, which is thought to contribute to retrograde atrophy of neurons whose axons are damaged (Kobayashi et al., 1997; Jones et al., 2001; Hains et al., 2003; Zhou and Snider, 2006); and 6) the lack of appropriate spatial and temporal gradients of growth factors and guidance cues necessary to stimulate and guide growing axons to their appropriate targets (Alto et al., 2009; Blesch and Tuszynski, 2009). Although the relative contribution of the various growth inhibitory elements present in the CNS remains unclear, it was recently demonstrated that CSPGs produced by reactive astrocytes can act via Nogo receptors (NgR1 and NgR3), indicating that CSPGs and myelin proteins may at least partly use shared mechanisms to exert their inhibitory effects (Dickendesher et al., 2012). In addition, to myelin proteins and CSPGs, myelin and the glial scar are also known to contain other inhibitory guidance molecules, such as RGM (Schwab et al., 2005a; Schwab et al., 2005b; Hata et al., 2006), ephrins/Ephs (Goldshmit et al., 2004; Benson et al., 2005), semaphorins (Moreau-Fauvarque et al., 2003; Kantor et al., 2004); netrins (Löw et al., 2008) and slits (Wehrle et al., 2005), all of which are known/thought to contribute to the inhibition of CNS axon regeneration (Schwab et al., 2006; Cafferty et al., 2010).
The lack of regeneration following SCI eventually leads to atrophy of the cell bodies of many long projecting axons that reside in the brain or in the spinal cord below the level of injury, despite the fact that those neuronal somata are well removed from the site of injury (Kobayashi et al., 1997; Kwon et al., 2002b). Indeed, in humans, SCI is often associated with notable atrophy of the primary motor and sensory cortices (Freund et al., 2011). Neuronal atrophy leads to decreased responsiveness to growth factors and other treatments delivered to the site of injury, which makes promoting regeneration increasingly difficult in the chronic setting (Kwon et al., 2004). However, not all neurons atrophy following injury, and in contrast to the situation at the site of injury, spontaneous growth and sprouting of both injured and uninjured CNS axons has been found to occur after incomplete SCI. For example, following hemisection or discrete transection of the corticospinal tract (CST), spontaneous plasticity in the CST or rubrospinal tract (RST) has been found to occur, resulting in the formation of new neural circuits that bypass the lesion site in mice (Steward et al., 2008), rats (Raineteau et al., 2002; Bareyre et al., 2004), and monkeys (Rosenzweig et al., 2010). This plasticity is thought to play a key role in the spontaneous, and often substantial, recovery of locomotor function that can occur following incomplete SCI in both animals and humans (Little and Halar, 1985; Roth et al., 1991; Rossignol et al., 1999; Courtine et al., 2005), and at least in rats, the transection of newly formed circuits has been shown to result in the loss of recovered electrophysiological and behavioral functions (Bareyre et al., 2004). In parallel with neuronal plasticity in the spinal cord, there is also a growing body of evidence that indicates substantial cortical plasticity occurs following SCI, particularly involving the functional reorganization of the sensorimotor cortex, in both rats and humans (Bareyre et al., 2004; Freund et al., 2011; Henderson et al., 2011).
1.3.4 **Long-term pathophysiological outcomes of SCI**

Although the initial spread of secondary damage is rapid, resulting in substantial and irreversible damage to the gray matter within 1 hour of injury and the white matter within 72 hours of injury following contusive SCI, the expansion of the lesion site slows over time and eventually stops (Blight and Young, 1989; Dumont et al., 2001). Neurogenic shock subsides and the vasculature stabilizes, leading to relatively normal tissue perfusion and adequate oxygen and glucose to once again support aerobic respiration (Dumont et al., 2001). Although the reperfusion of ischemic tissue is known to initially exacerbate the loss of cells due to reperfusion injury, more extensive cellular damage would likely occur if ischemia was left unchecked. The permeability of the BSCB returns to normal in intact regions by 1-2 months post-injury (Bartanusz et al., 2011), which helps to prevent further edema. Edema itself eventually subsides after SCI, although precisely how that occurs remains a mystery, as little is known about the mechanisms of excess fluid elimination from the spinal cord (Saadoun and Papadopoulos, 2010). While the initially massive cellular inflammatory response eventually subsides, macrophages and reactive microglia are often found in the spinal cord months and even years after injury – particularly in regions of ongoing Wallerian degeneration (Schmitt et al., 2000; Popovich and Jones, 2003; Buss et al., 2004; Norenberg et al., 2004). As the production of free radicals decreases, it eventually reaches a point where the level of antioxidants is sufficient to maintain balance, thus reducing/eliminating oxidative stress. Excess levels of excitatory amino acids are eventually cleared from the extracellular space, as is myelin and other debris; although some portion of debris is commonly found in regions of ongoing Wallerian degeneration, where degenerating and severed axons with denuded segments persist after SCI, even decades later in
some human cases (Gledhill et al., 1973a; Norenberg et al., 2004; Guest et al., 2005; Totoiu and Keirstead, 2005).

In most, if not all types of SCI, secondary injury actually results in more tissue damage than primary injury (Hausmann, 2003). The combined result of those two processes is often the loss of a substantial portion of tissue, as indicated by a 30% reduction in cord area according to MRI in human SCI patients; indicative of gross atrophy of the spinal cord following injury (Freund et al., 2011). In injuries involving contusion/compression (including dislocation), primary and secondary damage typically culminate in the formation of an oval-shaped fluid-filled cyst/cavity that extends 2-3 spinal segments surrounded by an inhibitory glial scar and a rim of spared/intact white matter that is far from normal, as it contains a reduced density of axons, particularly those with large (≥ 5µm) diameter, abnormal myelin sheaths (due to remyelination), and often contains macrophages, and myelin debris (Blight, 1985, 1991, 1992; Beattie et al., 1997; Shuman et al., 1997; Bunge, 2001; Silver and Miller, 2004; Rosenberg et al., 2005; Dietz and Curt, 2006) The extent of damage following contusion typically includes a degree of peripheral degeneration, which is thought to contribute to the resultant functional deficits, but commonly receives little attention in experimental treatment paradigms (Dietz and Curt, 2006; Van De Meent et al., 2010).

In contrast to contusion injuries, transections/hemisections of the spinal cord are characterized by limited rostrocaudal spread of secondary injury, and thus tend to result in a much smaller lesion overall (Hausmann, 2003) and much less extensive demyelination (Siegenthaler et al., 2007). In addition, transections, hemisections, and crush injuries limited to specific tracts (e.g., dorsolateral funiculus crush used here) all involve damage to the dura and
meninges, and thus allow for more rapid and increased invasion by peripheral monocytes/macrophages, as well as extensive infiltration of the spinal cord by cells that are normally restricted to the meninges, such as fibroblasts (Hausmann, 2003; Norenberg et al., 2004); the latter of which create a mesenchymal/fibroblastic scar composed mainly of collagen that impedes axonal growth even more than the astroglial scar (Berry et al., 1983).

In the face of the extensive loss of tissue caused by SCI, the delay in cell replacement (despite the presence of endogenous spinal cord stem/progenitor cells), and the failure of CNS axonal regeneration, most of the damage caused by SCI is permanent, and thus results in persistent functional deficits, the range and extent of which are determined by the particular neural circuits left disrupted or dysfunctional after injury (Dietz and Curt, 2006). However, some degree of spontaneous functional recovery is common in the weeks and months following SCI in both animals and humans (Onifer et al., 2011), and is primarily thought to occur via three mechanisms: 1) the resolution of spinal shock (Holaday and Faden, 1983; Hiersemenzel et al., 2000); 2) remyelination of denuded intact/spared axons (Blight and Young, 1989; Gensert and Goldman, 1997); 3) the creation of new functional neural circuits via the sprouting of injured/uninjured axons both in the spinal cord, brainstem, and cortex (Bareyre et al., 2004; Bareyre, 2008). Although spontaneous recovery can be substantial following incomplete SCI, particularly for less severe injuries, even with the addition of modern surgical and rehabilitative interventions, recovery eventually plateaus in all patients, typically within the first year (Lorenz et al., 2012), so the vast majority of people with SCI are left with significant functional impairments and lifelong disability.
1.4 Therapeutic strategies for promoting improved neurological outcome after SCI

There is nothing that can be done to stop the immediate damage produced by the initial trauma that causes SCI, so research is generally focused on developing treatments that act to limit the spread of secondary damage (i.e., neuroprotective strategies) and/or promote tissue repair (i.e., neurorepair strategies) during or subsequent to secondary injury. The most promising treatments for SCI are generally those that provide multiple neuroprotective and/or neuroreparative benefits, and cellular transplantation is one such multidimensional therapeutic strategy that has garnered considerable attention in the field of SCI (for reviews see: Bunge and Pearse, 2003; Tator, 2006; Fehlings and Vawda, 2011; Tetzlaff et al., 2011; Mothe and Tator, 2012; Ruff et al., 2012).

1.4.1 Neuroprotective strategies for SCI

Examples of neuroprotective strategies tested in animals models or humans with SCI include: 1) surgical decompression and spinal stabilization to minimize physical tissue damage (Dolan et al., 1980; Guha et al., 1987; La Rosa et al., 2004; Carreon and Dimar, 2011); 2) management of blood flow to minimize ischemia (Guha et al., 1989; Tator, 1992); 3) preservation of vascularity (e.g., promotion of endothelial survival) to minimize hemorrhage and edema (Han et al., 2010; Lutton et al., 2012; Simard et al., 2012); 4) prevention or reversal of membrane and cytoskeletal damage to limit axonal degeneration, cell death and/or atrophy (Borgens and Shi, 2000; Schumacher et al., 2000); 5) attenuation of inflammation to limit bystander damage (Xu et al., 1998; Gris et al., 2004; Stirling et al., 2004; Geremia et al., 2012); 6) boosting antioxidant levels or minimizing the generation of free radicals to limit oxidative damage (Diaz-Ruiz et al., 1999; Bains and Hall, 2012); 7) blocking glutamate receptors to
prevent excitotoxicity (Faden et al., 1988; Wrathall et al., 1996; Gaviria et al., 2000); 8) preventing cell death and/or atrophy by boosting neurotrophin levels (Giehl and Tetzlaff, 1996; Hammond et al., 1999; Iannotti et al., 2004; Awad et al., 2013; Zhang et al., 2013) or blocking cell death directly (Ozawa et al., 2002; Yune et al., 2008); 9) early remyelination to prevent delayed axonal regeneration (Irvine and Blakemore, 2008).

Neuroprotective interventions for SCI most often involve non-invasive systemic drug treatments that are generally administered as soon as possible after injury, in the hopes of attenuating as much secondary damage as possible (Kwon et al., 2011a). Systemic treatments are non-invasive and can thus be administered rapidly following injury, but these treatments are often associated with unwanted side effects, which can be severe enough to outweigh the neurological benefits of a given drug. The corticosteroid methylprednisolone is a prime example of this, as high doses of methylprednisolone provide modest improvement in neurological function when delivered early after SCI (Bracken et al., 1990; Bracken et al., 1992; Bracken et al., 1997; Bracken et al., 1998), but many clinicians do not consider those effects to be robust enough to outweigh the increased risk of potentially deadly infections observed in clinical trials, so although the use of methylprednisolone was once widely adopted, it is no longer considered a standard of care for acute SCI (Pointillart et al., 2000; Hurlbert and Hamilton, 2008; Lammertse, 2013). Potential neuroprotective therapies that address early mechanisms of secondary damage also often have extremely limited therapeutic windows, thus making early intervention not just preferable, but actually essential to treatment efficacy. For example, a variety of drugs have demonstrated efficacy only if delivered within hours of injury, including: 8-hrs for methylprednisolone (Bracken et al., 1990), 2-hrs for the PPAR (peroxisome proliferator-
activated receptor) agonist pioglitazone (Park et al., 2007), 1-hr for polyethylene glycol (Baptiste et al., 2009), <2-hrs for riluzole (Mu et al., 2000). Although systemic drug treatments are the most common form of neuroprotective intervention, treatments delivered directly to the injured spinal cord, including both cellular and non-cellular (e.g., the Rho antagonist cetherin) approaches can also exert neuroprotective effects if delivered early enough following injury (Kwon et al., 2011a; Kwon et al., 2011b; Tetzlaff et al., 2011).

1.4.2 Neurorepair strategies for SCI

Whereas the goal of all neuroprotective strategies is to prevent the loss of neurological functions by minimizing the death/atrophy of neural cells and/or the degeneration/demyelination of axons following SCI, the goal of all neurorepair strategies is to facilitate the return of lost neurological functions by promoting axonal regeneration or plasticity in spared/injured neural circuits, and/or replacing lost cells; the latter of which is most often focused on replacing lost neurons to form new neural circuits or replacing oligodendrocytes to facilitate remyelination and the return of rapid axonal conduction.

A wide range of therapeutic approaches have been used to promote axonal regeneration/plasticity following SCI, including: 1) the reversal of neuronal atrophy via neurotrophin delivery to neuron cell bodies (Hiebert et al., 2002; Kwon et al., 2002b); 2) boosting the intrinsic growth capacity of CNS axons via intracellular pathways (e.g., cyclic adenosine monophosphate [cAMP] or phosphatase and tensin homologue [PTEN]) (Nikulina et al., 2004; Liu et al., 2010); 3) delivering exogenous neurotrophins at the site of injury (Grill et al., 1997; Kobayashi et al., 1997; Vavrek et al., 2006; Kwon et al., 2007); 4) electrical stimulation (Borgens et al., 1987; Shapiro et al., 2005); 5) removal/degradation of inhibitory
CSPGs (Yick et al., 2000; Bradbury et al., 2002); 6) blocking myelin-associated inhibition (GrandPre et al., 2002; Fouad et al., 2004; Liebscher et al., 2005; Freund et al., 2006; Cao et al., 2008; Freund et al., 2009); 7) preventing growth cone collapse (e.g., blocking activation of Rho kinase) (Dergham et al., 2002; Fournier et al., 2003; Fehlings et al., 2011); 8) using exercise and physical rehabilitation to boost endogenous neurotrophin (Gomez-Pinilla et al., 2001; Van Meeteren et al., 2003); 9) the transplantation of synthetic or biological conduits that bridge or bypass the lesion site (Rochkind et al., 2006; Li et al., 2009; Macaya and Spector, 2012); and 10) the transplantation of cells or tissues that bridge the lesion site and/or produce factors (e.g., neurotrophins) that promote axonal growth, including: peripheral nerve grafts (David and Aguayo, 1981; Cheng et al., 1996; Levi et al., 2002), fetal spinal cord tissue (Houle and Reier, 1988; Reier et al., 1988; Jakeman and Reier, 1991; Diener and Bregman, 1998), fibroblasts (Grill et al., 1997; Jin et al., 2002), bone marrow stromal cells (BMSCs) or bone marrow-derived stem cells (mesenchymal and/or hematopoietic stem cells [MSCs and HSCs, respectively]) (Hofstetter et al., 2002; Wu et al., 2003; Himes et al., 2006; Zurita and Vaquero, 2006), olfactory ensheathing glia (OEG) (Ramon-Cueto et al., 1998; Imaizumi et al., 2000; Ramer et al., 2004c; Richter et al., 2005; Andrews and Stelzner, 2007), or N-SCs (Xu et al., 1997; Bunge, 2001; Pearse et al., 2004a; Barakat et al., 2005; Andrews and Stelzner, 2007; Schaal et al., 2007).

Although many of those treatments have been found to significantly bolster neural plasticity and sprouting in animal models of SCI, most largely fail to produce growth that would be characterized as ‘regeneration’, and even for those that do (e.g., PTEN deletion), the degree of regeneration often remains of questionable neurological significance (Illis, 2012). Complicating this matter further is the fact that different neuronal populations in the spinal cord tend to
respond to injury/treatment differently, as dorsal root ganglion sensory axons and propriospinal axons regenerate much more readily in response to treatments than supraspinal axons, and the most important tract in terms of voluntary motor control in humans, the corticospinal tract, is generally the most resistant to regenerative therapies (Blesch and Tuszynski, 2009; Hawthorne et al., 2011).

In theory cellular replacement and remyelination are two neuroreparative strategies that could be accomplished by promoting the proliferation, migration, and appropriate differentiation of endogenous NSPCs or OPCs. However, as discussed previously, those precursors generally fail to produce neurons following SCI and the endogenous response is clearly insufficient to replace all of the astrocytes and oligodendrocytes that are lost to injury. Although alternative strategies to promote endogenous cellular replacement and remyelination are in development (Mi et al., 2007; Mi et al., 2009; Fancy et al., 2011; Huang et al., 2011), for the time being the transplantation of exogenous cells is the standard therapeutic approach used to attain those goals both in humans and animals following SCI. Partial replacement of lost CNS neurons has been reported following the transplantation of fetal spinal cord tissue (Reier et al., 1986; Bregman et al., 1993), fetal CNS neurons (Privat et al., 1989), or NSPCs from fetal spinal cord (Ogawa et al., 2002; Lepore et al., 2006; Yan et al., 2007), brain (Cummings et al., 2005; Abeinatsu et al., 2010; Salazar et al., 2010), and embryonic stem (ES) cells or induced pluripotent stem (iPS) cells pre-differentiated towards a neural fate (McDonald et al., 1999; Tsuji et al., 2010; Nori et al., 2011; Fujimoto et al., 2012). Recent progress in cellular transdifferentiation now allows for the direct conversion of mouse and human skin or liver cells into functional neurons (termed induced neuronal cells) via the forced ectopic expression of neural lineage-specific transcription
factors (Vierbuchen et al., 2010; Ambasudhan et al., 2011; Marro et al., 2011). That approach has even been used to specifically generate spinal motor neurons from embryonic mouse and human fibroblasts (Son et al., 2011), but none of those induced neuronal cells have been examined as treatments for SCI as of yet. Lost oligodendrocytes can also be replaced after SCI and this has been accomplished to varying degrees following the transplantation of NSPCs from the adult/fetal spinal cord (Ogawa et al., 2002; Hofstetter et al., 2005; Mothe et al., 2008; Mothe and Tator, 2008) or brain (Cummings et al., 2005; Karimi-Abdolrezaee et al., 2006; Karimi-Abdolrezaee et al., 2010; Salazar et al., 2010), NSPCs generated from ES/iPS cells (McDonald et al., 1999; Tsuji et al., 2010; Nori et al., 2011), glial restricted precursors from fetal spinal cord (Han et al., 2004; Cao et al., 2005; Lepore and Fischer, 2005), or OPCs isolated from neonatal/adult brain or spinal cord (Bambakidis and Miller, 2004; Lee et al., 2005; Cao et al., 2010) or differentiated from ES cells (Keirstead et al., 2005; Sharp et al., 2010) or iPS cells (Czepiel et al., 2011). Myelinating OPCs have even been generated from cultured embryonic/fetal neural crest stem cells, which only generate peripheral neurons and glia during normal development (Binder et al., 2011).

It is important to note that neuronal and oligodendroglial differentiation of transplanted stem/progenitor cells is often difficult to attain, as most of the NSPCs that can be isolated from the mammalian CNS preferentially produce astrocytes following transplantation into the injured spinal cord and this pronounced astroglial differentiation has been associated with the development of allodynia in rats with SCI (Akiyama et al., 2001; Cao et al., 2001; Vroemen et al., 2003; Hofstetter et al., 2005). As such, it is not uncommon for NSPCs to be pre-differentiated toward the desired fate(s) \textit{in vitro} prior to transplantation or delivered in conjunction with co-
treatments that help to guide appropriate differentiation \textit{in vivo} (Cao et al., 2005; Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006; Cao et al., 2010; Karimi-Abdolrezaee et al., 2010). Furthermore, the survival of many of those cell types is poor, particularly when they are transplanted directly into the lesion cavity, so NSPCs and their progeny are often transplanted into intact parenchyma adjacent to the lesion site in combination with co-treatments aimed at enhancing the survival of transplanted cells (Karimi-Abdolrezaee et al., 2006; Parr et al., 2007; Karimi-Abdolrezaee et al., 2010). In addition to oligodendrocyte replacement, remyelination can be enhanced by transplanting N-SCs, which are known to remyelinate CNS axons and increase the presence of endogenous remyelinating SCs (Hill et al., 2006; Hill et al., 2007; Pearse et al., 2007). Remyelination is also enhanced by OEG and BMSC/MSC transplants, and although the ability of those cells to directly myelinate axons remains a matter of some debate, they are both known to promote remyelination by significantly increasing the presence of endogenous SCs in the injured spinal cord (Ramer et al., 2004c; Lu et al., 2005; Lu et al., 2007; Pearse et al., 2007).

\subsection{1.4.3 The need for combinatorial treatments}

Although many potential neuroprotective treatments have been shown to significantly attenuate secondary damage in animal models of SCI, none of them completely prevent the loss of cells/tissue due to secondary injury, and the resulting neurological benefits associated with a single treatment are often only modest at best (for review see Kuffler, 2012; Tator et al., 2012). Regenerative approaches have commonly yielded similar results, as significant increases in axonal regeneration, neuronal plasticity, remyelination, and/or neuronal replacement in animal models of SCI are commonly associated with significant functional improvements, but those neurological effects are generally modest unless multiple treatments are combined (Fouad et al.,
2005; Blesch and Tuszyński, 2009; Tetzlaff et al., 2011). In light of all that, and given the myriad of inter-related pathological mechanisms underlying secondary injury in SCI and the number of potential targets for neurorepair (e.g., axonal regeneration/plasticity, cavity/gap bridging, remyelination), it is widely recognized that a combination of multiple complimentary co-treatments (i.e., a combinatorial approach) aimed at both neuroprotection and repair/regeneration will be required to maximize neurological recovery after SCI in the clinic (Bunge, 2001; Ramer et al., 2005; Bunge, 2008; Oudega et al., 2012). As such, the focus of SCI treatment research has broadened over time, to include not only the search for novel efficacious treatments, but also on the testing of combinatorial therapies that utilize multiple complementary treatments to maximize neurological recovery. While this approach is common in preclinical work, it has yet to be adopted in human clinical trials because the translation of combinatorial therapies requires that the safety (and ideally efficacy) of each experimental co-treatment be established independently, which makes gaining regulatory approval for combinatorial therapies much more difficult and expensive than it is for single therapies (Tetzlaff et al., 2011). Thus, from a regulatory standpoint, it is desirable to minimize the number of treatments that need to be combined to produce efficacious outcomes after SCI, and in this regard it seems wise to focus on treatments that are known to provide multiple beneficial neuroprotective and/or regenerative effects.

Cellular transplantation is a prime example of such a treatment, as unlike most pharmacological / biological treatments that are specifically designed to target a single mechanism of secondary damage or repair, multiple mechanisms of therapeutic action are the norm for most cellular therapies. For example, cells commonly produce a variety of growth
factors, neurotrophins, cytokines, chemokines, and growth permissive extracellular matrix components and some cells provide additional benefits (e.g., creating growth permissive bridges across cavities/gaps in tissue, the replacement of lost neurons or glia, and remyelination) that generally cannot be effectively replicated using non-cellular approaches at present. In light of these characteristics, cellular therapies have garnered considerable attention as potential treatments for SCI over the last two decades, and are considered by many researchers to be a very likely component of any successful combinatorial therapy for SCI (Bunge, 2001; Murray and Fischer, 2001; Ramer et al., 2005; Bunge, 2008; Kadoya et al., 2009; Tetzlaff et al., 2011; Oudega et al., 2012).

1.5 Cellular therapies for SCI

Transplantation has a long history in preclinical studies of CNS repair, dating back to the seminal work of Tello (1911) and Ramón y Cajal (1928), who transplanted pieces of peripheral nerve into the cortex and spinal cord and described the growth of presumptive CNS axons into those grafts (reviewed in: Aguayo et al., 1981; Oudega and Xu, 2006; Bunge and Wood, 2012). However, the identity of those growing fibres was contested (Le Gros Clark, 1943), and remained so until 1980, when Aguayo and colleagues final provided definitive evidence that axons of central origin did indeed regenerate into peripheral nerve (PN) grafts (Richardson et al., 1980). That work was followed closely by the first transplantation of a purified population of cells into the injured spinal cord, which involved the transplantation of nerve-derived SCs (N-SCs) into regions of focal demyelination produced by injections of lysolecithin (Duncan et al., 1981), and the transplantation of fetal spinal cord tissue following traumatic SCI (Reier et al.,
1986). Although the work of Duncan et al. (1981) did not involve traumatic injury of the CNS, it represents an important step in the evolution of cell-based strategies, as modern transplantation paradigms typically involve the *in vitro* expansion and purification of cells prior to application, rather than the use of tissue grafts; although the latter approach continues to be examined as a therapy for CNS repair (Cheng et al., 2004; Tsai et al., 2005; Tuszynski, 2007).

Since the inception of transplantation-based therapies for SCI a wide variety of transplant candidate cells/tissue have been examined using animal models of traumatic injury. Some of the more popular/promising cellular therapies include: 1) fetal spinal cord tissue (Reier et al., 1986; Houle and Reier, 1988; Reier et al., 1988; Jakeman and Reier, 1991; Bregman et al., 1993; Giovanini et al., 1997); 2) peripheral nerve grafts (Cheng et al., 1996; Dolbeare and Houle, 2003; Cheng et al., 2004; Tsai et al., 2005; Tom et al., 2009); 3) NSPCs harvested from the brain or spinal cord, including neural stem cells (NSCs), neural- or glial- restricted precursors (i.e., NRPs or GRPs) and OPCs (Ogawa et al., 2002; Bambakidis and Miller, 2004; Cao et al., 2005; Cummings et al., 2005; Lee et al., 2005; Mitsui et al., 2005; Karimi-Abdolrezaee et al., 2006; Mothe et al., 2008; Abematsu et al., 2010; Cao et al., 2010; Karimi-Abdolrezaee et al., 2010; Salazar et al., 2010); 4) NSPCs generated from ES or iPS cells (Keirstead et al., 2005; Sharp et al., 2010; Tsuji et al., 2010; Nori et al., 2011); 5) olfactory cells/tissue, including olfactory ensheathing cells/glia (OECs or OEG) and olfactory mucosa tissue (Li et al., 2003; Ramer et al., 2004b; Richter and Roskams, 2007; Toft et al., 2007; Bretzner et al., 2010; Granger et al., 2012); 6) stem/progenitor cells from non-neural tissue including bone marrow stromal cells (BMSCs), mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and adipose stem cells (ASCs) (Hofstetter et al., 2002; Wu et al., 2003; Himes et al., 2006; Zurita and Vaquero, 2006;
Busch et al., 2011; Wright et al., 2011); and 7) N-SCs (Xu et al., 1997; Pearse et al., 2004a; Barakat et al., 2005; Andrews and Stelzner, 2007; Schaal et al., 2007; Flora et al., 2012; Ghosh et al., 2012). A thorough review of the advantages and disadvantages of each of these cell types is well beyond the scope of this dissertation, but the reader is directed to a number of recent and highly informative reviews on this topic for further information (for reviews see: Bunge and Pearse, 2003; Tator, 2006; Eftekharpour et al., 2008; Fehlings and Vawda, 2011; Tetzlaff et al., 2011; Harrop et al., 2012; Mothe and Tator, 2012; Ruff et al., 2012; Sandner et al., 2012).

Although the particular benefits provided vary substantially depending on the cell type in question, commonly reported benefits following cellular transplantation after SCI include: neuroprotection, immunomodulation, bridging of lesion cavities/gaps, remyelination, facilitation of axonal regeneration or plasticity, and/or the replacement of lost neurons (Fehlings and Vawda, 2011; Tetzlaff et al., 2011; Mothe and Tator, 2012). Many of those benefits are thought to be related to the production of growth factors, neurotrophins, cytokines, chemokines, and growth permissive extracellular matrix components, as transplanted cells naturally produce a variety of those factors (for examples see: Caplan and Dennis, 2006; Oudega and Xu, 2006; Richter and Roskams, 2007). Provided that the cells survive transplantation and persist long-term, they may produce those factors indefinitely; thereby providing sustained and localized delivery of multiple potentially therapeutic molecules simultaneously (Tuszynski, 2002). Indeed, for some cell types (e.g., BMSCs, MSCs, and HSCs), the production of multiple growth factors and cytokines is thought to be the primary mode of efficacy (Caplan and Dennis, 2006; Nishio et al., 2006; Meirelles Lda et al., 2009; Quertainmont et al., 2012). However, the multidimensional nature of cellular therapies often makes it difficult to definitively attribute beneficial effects to one
particular mechanism of action, particularly when transplanting cells that integrate with spared neural tissue in the injured CNS to replace lost neurons/glia. The ability of cells to provide localized and potentially sustained therapeutic delivery can also be taken advantage of by using cells as a vector for gene therapy (reviewed by: Tuszynski, 2002; Blesch et al., 2012). Examples of this are replete in the literature, as cells are often genetically modified to over-express particular neurotrophins prior to transplantation, and this approach has been used repeatedly to enhance the regenerative efficacy of cellular therapies in animal models of SCI (for example see: Grill et al., 1997; Tuszynski et al., 1998; Weidner et al., 1999; Jin et al., 2002; Murray et al., 2002; Golden et al., 2007).

Although there are many potential benefits to cellular transplantation as a treatment for SCI, there are also limitations inherent to cellular therapy. Cells must be isolated from tissue, and the source of tissue used to generate cells for transplantation has important implications for the potential clinical application of a given cellular therapy. Cells cannot be harvested directly from the CNS without causing injury to the brain or spinal cord, and thus CNS-derived neurons, oligodendrocytes, and NSPCs/OPCs cannot be directly isolated from autologous sources or living donors for the purposes of therapeutic transplantation. Instead, these cells must be harvested from cadaveric adult tissue or aborted fetuses, or generated from alternative tissue sources such as ES cells or iPS cells. Mature neurons and oligodendrocytes are relatively fragile cells that generally do not survive well when isolated, purified and transplanted into the hostile environment of the injured spinal cord, and those cells are post-mitotic, so large amounts of tissue would be necessary to generate sufficient cells for therapeutic application. NSPCs and OPCs provide an attractive alternative to mature cells because they can be expanded from a
small amount of tissue, however, cadaveric adult tissue is not ideal as a source for those cells because it is difficult to expand adult-derived cells to clinically sufficient numbers (Mothe & Tator, 2012).

NSPCs generated from fetal or embryonic sources do not suffer that limitation, as they expand readily under appropriate culture conditions, but the application of cells derived from both of those sources is mired by ethical concerns regarding the use of aborted human tissue (Fehlings and Vawda, 2011; Sandner et al., 2012). The generation of NSPCs from iPS cells obviates ethical concerns associated with the use of cells derived from fetal/embryonic sources, as iPS cells can be generated from adult autologous tissue sources (usually skin fibroblasts). However, both ES cells and iPS cells require thorough in vitro pre-differentiation because undifferentiated cells will form teratomas after transplantation (Thomson et al., 1998; Reubinoff et al., 2000; Tsuji et al., 2010; Czepiel et al., 2011), and thus ES- or iPS- derived NSPCs carry an increased risk of tumorigenesis. Recently developed protocols for the direct reprogramming of embryonic rodent skin cells into NSPCs, known as induced neural precursor cells [iNPCs] (Lujan et al., 2012), appears to obviate both of those concerns, as these cells can be generated from autologous tissues with minimal risk of tumorigenicity (Masip et al., 2010), but it remains to be seen whether iNPCs can be generated from adult human fibroblasts and these cells have yet to be studied as transplantation candidates for the treatment of SCI.

The use of autologous cells for transplantation therapies is advantageous because it eliminates concerns about tissue sourcing, but also because it circumvents the need to use immunosuppressive drugs (e.g., cyclosporine A [CsA]) to prevent the rejection of transplanted cells. The CNS is normally considered an ‘immune privileged’ organ, partly because the BBB
and BSCB tend to restrict the entry of adaptive (e.g., T cells) and innate (e.g., macrophages) immune cells from the circulation into intact CNS parenchyma, but also because the CNS lacks a cellular pathway for the delivery of antigens to regional lymph nodes; which typically occurs robustly in other tissues and is required to prime and activate T cells for the adaptive immune response (Galea et al., 2007). Instead, only soluble antigens normally escape the CNS, via drainage of the lymph into local nodes, which is relatively sparse in the CNS (Barker and Widner, 2004). The destruction of the vasculature and the breakdown of the BSCB after SCI cause the loss of CNS immune privilege, as circulating immune cells gain access to the injured spinal cord parenchyma and peripherally-derived macrophages are then free to act as antigen presenting cells in local lymph nodes. In this environment, transplanted allogeneic cells are a prime target for T cell-mediated rejection, and thus the use of immunosuppressant drugs is typically required to safeguard transplanted cells that are not autologously derived. Although the CNS eventually regains immune privilege following injury, as demonstrated by the long-term survival of allogeneic cells grafted into the CNS of rats and humans following the withdrawal of immunosuppression (Duncan et al., 1981; Kordower et al., 1995; Theele and Reier, 1996; Wirth et al., 2001), there is some evidence that slow chronic rejection may occur following the withdrawal of immunosuppression in humans transplanted with embryonic neural tissue (Barker and Widner, 2004). Furthermore, immunosuppression has been applied for at least 6 months (and more often 12-18 months) in human clinical trials conducted to date (Wirth et al., 2001; Barker and Widner, 2004), so relatively long-term immunosuppression remains standard for non-autologous cellular therapies in the clinic.
Unfortunately, the prolonged use of non-specific, systemic immunosuppression following organ transplantation in humans is well known to contribute to morbidity and mortality among transplant recipients (Denton et al., 1999). Besides the obvious increase in the risk of infection, long-term use of immunosuppressant drugs is regularly associated with kidney and liver damage due to drug toxicity, increased rates of malignancy, and increased risk of cardiovascular and metabolic diseases (reviewed by: Denton et al., 1999; Whitlock et al., 2010; Lallana and Fadul, 2011) in that population. As such, avoiding the need for immunosuppression by transplanting autologous cells is preferable. However, it should be noted that not all autologous sources of cells are guaranteed to be non-immunogenic. For example, recent evidence indicates that undifferentiated mouse iPS cells are susceptible to immune-mediated rejection even if they are transplanted from autologous sources (Zhao et al., 2011). This unexpected finding has yet to be extended to autologous iPS cells that have been differentiated into therapeutically relevant phenotypes, but suggests that immunogenic testing of iPS-derived cells should be conducted prior to human clinical application (Apostolou and Hochedlinger, 2011), and this testing should likely be extended to any transdifferentiated cells including iNPCs if/when those cells are generated from human tissue.

Over the last couple of decades, the lack of readily available autologous sources of NSPCs/OPCs for therapeutic application has prompted many researchers to examine the efficacy of autologous cells harvested from non-CNS sources. Tissues from outside of the CNS typically display a much greater capacity for regeneration in adult mammals, and cells can be harvested from a variety of autologous sources with only relatively minor functional consequences. Indeed, many tissues contain cell types that can be harvested, purified, and expanded in vitro for
transplantation into the injured spinal cord, including non-neural cells such as BMSCs, MSCs, HSCs, and ASCs harvested from bone marrow, blood and/or adipose tissue, OECs/OEGs harvested from the olfactory bulb or lamina propria, and N-SCs harvested from the peripheral nerve. For some of these cell types (e.g., MSCs and HSCs), claims of spontaneous or direct neural transdifferentiation have been reported, but those results have now largely been refuted (Vitry et al., 2003; Lu et al., 2004; Neuhuber et al., 2004; Krabbe et al., 2005). Attempts have also been made to transdifferentiate M-SCs from a variety of sources into SC-like cells in efforts to develop alternative sources of autologous SCs for therapeutic application; a topic which is discussed in detail below (see Section 1.7.1).

Isolated cells are typically purified to remove unwanted cell types and expanded in vitro prior to transplantation. Most transplantation paradigms utilize millions of cells, so adequate expansion can take anywhere from a week to a couple of months, depending on the proliferation rate of the cells in question, the amount of starting material, and whether or not the cells need to be pre-differentiated prior to purification/expansion or transplantation (e.g., NSPCs, ES cells, iPS cells). In addition, the transplantation of most cell types requires direct delivery into the spinal cord parenchyma to be effective, which imposes a minor delay in intervention timing because direct delivery of the cells necessitates invasive surgery to expose the injured spinal cord. Although the delay imparted by cell culturing might be minimized by administering ‘ready-to-use’ cellular treatments (e.g., the use of immortalized cell lines, or pre-made frozen batches of cells), which may be commercially available in the future, the delay of treatment due to surgery is generally unavoidable. As a result of these conditions, the delivery of cellular therapies will
always be delayed somewhat compared to systemic pharmacological therapies, the latter of which are thus better suited for targeting very early events in secondary pathology.

To date, many transplant candidate cells have demonstrated varying degrees of therapeutic benefit in animal models of SCI, and no single transplant candidate cell type has emerged as the clear choice for transplantation-based repair of the injured adult mammalian spinal cord (Tetzlaff et al., 2011). Thus, after decades of research, the focus of research has shifted, from finding the ideal cell, to finding ways to optimize the therapeutic benefits that can be derived from transplantation via co-treatments. This combinatorial approach is widely recognized as the strategy most likely to provide successful treatments in the future, so modern transplantation studies tend to include: multiple cell types, genetically manipulated cells, and/or co-treatments with factors that promote neuroprotection, graft survival, and/or axonal regeneration (Reier, 2004). Despite this shift in attention, the identification of novel cell types and sources of cells that may be suitable for CNS transplantation remains a promising avenue of research. Herein we examine two novel cell types, as prior to our work in Chapter 2, neither SKPs nor SKP-SCs had ever been studied in the context of SCI. Although the transplantation of SKP-SCs into the CNS was novel, the notion that SCs were suitable for transplantation into the injured CNS was definitely not, as SCs were one of the first candidates cells studied in early CNS transplantation research, and to this day they remain one of the most promising cell types for therapeutic intervention following SCI (Raisman, 2001; Bunge and Pearse, 2003; Oudega et al., 2005; Odorfer et al., 2007; Oudega, 2007; Bunge, 2008; Tetzlaff et al., 2011).
1.6 Schwann cells

SCs are the myelinating glia of the PNS, and much like the oligodendrocytes of the CNS, SCs are absolutely essential for the maintenance of axon integrity, neuronal survival, and normal neurological functioning in the periphery (Jessen and Mirsky, 2005; Nave and Trapp, 2008). In addition, and unlike the oligodendrocytes of the CNS, SCs are known to dedifferentiate and proliferate in response to peripheral nerve injury, and those cells play a key role in the success of spontaneous regeneration and associated functional recovery that commonly occurs in the PNS, following traumatic injury in adult mammals (Vargas and Barres, 2007; Gaudet et al., 2011). SCs are also an important component of peripheral nerve grafts, which are the gold standard for autologous repair of PNS defects in situations where spontaneous PNS regeneration fails (Evans, 2000; Hood et al., 2009), and endogenous SCs that appear in the CNS following trauma or demyelination, may even contribute to what little spontaneous repair and recovery occurs following injury to the adult mammalian CNS (Beattie et al., 1997). For all of these reasons, the transplantation of nerve-derived SCs (N-SCs) has received substantial consideration as a potential therapy for CNS repair, and as we shall see, that interest was well founded, because these cells not only come from potentially autologous sources, but they also possess the ability to bridge gaps in nervous tissue, as well as myelinate, and facilitate the growth of, CNS axons. As a result of those properties, N-SCs remain a cell of interest for promoting the repair of the injured/demyelinated CNS to this day.

One of the main goals of the present work was to determine the similarities and differences between SCs generated from SKPs and SCs generated from peripheral nerve in order to address the question of whether SKP-SCs are a suitable replacement for N-SCs for the
purposes of therapeutic CNS repair. To accomplish a thorough comparison of those cell types requires a great deal of knowledge about each, and so in this section I review all of the pertinent information regarding the development SCs, their functions under normal conditions and following PNS/CNS pathology, and the evidence for therapeutic efficacy of cultured N-SCs as a treatment for neurological disorders resulting from injury/disease.

1.6.1 The development of SCs in spinal nerves

SCs are the predominant cell type in PNS, accounting for 90% of the nucleated cells in the endoneurial space of the adult PNS (Campana, 2007). There are two major phenotypes of SCs in the adult PNS: myelinating SCs and non-myelinating (ensheathing) SCs (Jessen and Mirsky, 2003). As their name suggests, myelinating SCs form the multi-layered, membranous myelin sheaths found around large-calibre (>1 µm) motor and sensory axons that are spaced approximately 1 mm apart along those fibres in the PNS (Campana, 2007). In contrast, non-myelinating SCs form a variety of specialized phenotypes including: SCs that form Remak bundles with small diameter (<1 µm) axons in peripheral nerve trunks, terminal/perisynaptic SCs found at neuromuscular junctions, and SCs found within sensory transducers such as Pacinian or Meissner’s corpuscles (Griffin and Thompson, 2008). Although satellite cells of the sensory and autonomic ganglia are sometimes considered non-myelinating SCs as well (Griffin and Thompson, 2008; Armati and Mathey, 2013), there is significant evidence suggesting that these cells represent a distinct lineage of peripheral glia that segregate from presumptive SCs early in development (Zirlinger et al., 2002; Woodhoo et al., 2004; Woodhoo and Sommer, 2008). For the present purposes we are concerned only with the development and behaviour of SCs that myelinate or form Remak bundles with sensory and motor axons in the trunks of peripheral
nerves in the limbs (i.e., spinal nerves), as those are the cells typically isolated for transplantation into the injured spinal cord. The generation of those myelinating and non-myelinating SCs involves 3 developmental transitions: 1) the generation of Schwann cell precursors (SCPs) from neural crest cells (gliogenesis), 2) the differentiation of SCPs to form immature SCs, and 3) the maturation and differentiation of those cells to form mature SC phenotypes (Jessen et al., 1994; Dong et al., 1995; Dong et al., 1999).

1.6.1.1 The migratory neural crest: Origin of SCs in spinal nerves

All of the SCs in the PNS originate from embryonic neural crest cells, which emerge from the dorsal-most part of the neural tube as it closes and migrate extensively to take up residence in various tissues within the embryo during early development (Dupin et al., 2006). The migratory neural crest is a highly heterogeneous population of cells, including multipotent neural crest stem cells (NCSCs), a variety of intermediate progenitors of varying potency, and even some committed precursors, and in addition to SCs, those cells give rise to all of the neurons (except some cranial nerve sensory ganglia) and other glia of the PNS (e.g., satellite cells), melanocytes of the skin, endocrine cells of the thyroid and adrenal glands, and mesenchymal cells that form much of the craniofacial skeleton, dermis, and adipose tissue during embryonic development (reviewed by: Le Douarin and Dupin, 2003; Dupin et al., 2006). When and where lineage specification occurs in the neural crest appears to vary depending on the specific neural crest-derivatives in question (Crane and Trainor, 2006; Dupin et al., 2007; Harris and Erickson, 2007), but it is of interest to note that at least some multipotent NCSCs persist in peripheral tissues into adulthood, and some of those cells are known to be capable of self-renewal (Dupin et al., 2006; Woodhoo and Sommer, 2008). This is highly pertinent to the
present work, as recent evidence indicates that the SKPs found in the head/neck dermis of adult mammals represent a population of neural crest-derived stem cells present in the skin of adult mammals (Jinno et al., 2010).

1.6.1.2 The generation of Schwann cell precursors

The neural crest cells that give rise to neurons and glia that form the spinal nerves of the PNS (i.e., excluding the autonomic and enteric nervous systems) emerge early in development, arising from the trunk level and following the ventral migratory stream (Dupin et al., 2006; Adameyko et al., 2009). Initially those cells primarily generate neurons, as the presence of high levels of neurogenic signals (e.g., bone morphogenetic protein 2 [BMP2] and Wnt) in the local environment bias neural crest cells towards a neuronal fate by activating pro-neural transcription factors (e.g., Mash1 and neurogenin-2) that drive downstream neuronal-differentiation genes (Bertrand et al., 2002). As neural crest cells give rise to the neurons that constitute the early ganglia, those cells begin extending axons that form the early embryonic spinal nerves (Dupin et al., 2006), and those growing nerves are soon populated by SCPs that arise from neural crest cells that have not yet adopted a neuronal fate (Woodhoo and Sommer, 2008). The extracellular signals and mechanisms involved in the specification of neural crest cells to the SCP fate remain a mystery to this day, but the transcription factor Sox10 (sex determining region Y-related high mobility group-box-10), which is expressed in every cell of the SC lineage, is known to be required for that step in SC development (Britsch et al., 2001; Mirsky et al., 2008; Woodhoo and Sommer, 2008).

Prior to reaching their targets, growing embryonic spinal nerves are highly compact structures composed exclusively of axons and tightly associated SCPs, with no surrounding
connective tissue, blood supply, or protective covering (Jessen and Mirsky, 2005). SCPs are
found around the edges of nerves and within the larger nerve trunks, and these cells form
extensive sheet-like processes that surround large groups of axons (Jessen et al., 1994). SCPs are
also intimately associated with growth cones at the front of those developing nerves (Wanner et
al., 2006); although they are not actually required for the nerves to grow and reach their final
targets (Grim et al., 1992; Riethmacher et al., 1997; Woldeyesus et al., 1999). SCPs produce all
of the immature SCs in the developing nerve, and yet they are not restricted to a glial fate, as
recent evidence indicates that SCPs also generate the small population of endoneurial fibroblasts
that arise later in nerve development (Joseph et al., 2004; Jessen and Mirsky, 2005) as well as a
substantial number of skin melanocytes (Adameyko et al., 2009). It is of interest to note here,
that SCPs from embryonic rodent tissues have been studied as potential treatments following
focal demyelination and traumatic injury to the spinal cord (Woodhoo et al., 2007; Agudo et al.,
2008).

1.6.1.3  The generation of immature SCs

As growing axons reach and synapse with their target tissues SCPs begin differentiating
into immature SCs. This process appears to be under the control of a combination of extracellular
signals including neuregulin 1 (NRG1), endothelin, and Notch signalling (Brennan et al., 2000;
Birchmeier and Nave, 2008; Adameyko et al., 2009; Woodhoo et al., 2009), and the down-
regulation of the transcription factor AP2α (activating enhancer binding protein 2 alpha) is
thought to play a role here because the generation of immature SCs is delayed when SCPs are
forced to over-express that factor (Stewart et al., 2001). The conversion of SCPs to immature
SCs occurs from embryonic day (E) 15-17 in the rat and E13-15 in the mouse (Jessen et al.,
1994; Dong et al., 1999), and coincides with dramatic changes in nerve cytoarchitecture, including the initial formation of the perineurium, which defines the early endoneurial space that contains bundles of axons enveloped by immature SCs and/or SCPs, blood vessels, endoneurial fibroblasts and extracellular matrix (Ziskind-Conhaim, 1988; Jessen and Mirsky, 2005; Wanner et al., 2006; Woodhoo and Sommer, 2008). By E17/18 in the rat and E15/16 in the mouse virtually all of the SCPs have converted to immature SCs, which maintain a communal relationship with axons, enveloping irregular clusters of axons in “axon-Schwann cell bundles” (or “families”) that persist neonatally (Webster and Favilla, 1984; Jessen et al., 1994; Dong et al., 1999). Unlike SCPs, immature SCs appear to be committed to the SC lineage, as these cells are not known to generate any cell types other than myelinating and non-myelinating SCs (Jessen and Mirsky, 2005). Immature SCs are the first cells in the SC lineage to produce a basal lamina, which is deposited around the entire axon-SC bundle at this stage, rather than around individual axons or small groups of axons as is typical for mature myelinating or non-myelinating SCs, respectively (Berti et al., 2011). These cells are also the first in the SC lineage to develop autocrine survival circuits by producing factors such as insulin-like growth factor (IGF), neurotrophin-3 (NT-3) and platelet-derived growth factor (PDGF), which can bind to appropriate receptors on the SCs to enable those cells to survive in the absence of axon-derived NRG1 (Dong et al., 1995; Dong et al., 1999; Meier et al., 1999; Jessen and Mirsky, 2005).

1.6.1.4 The generation of mature SCs

The transition from immature to mature SC phenotypes involves a rather complex process known as radial sorting, which begins prior to birth and continues postnatally (Woodhoo and Sommer, 2008). During radial sorting, immature SCs extend cytoplasmic processes into their
axon bundles. When those processes contact large diameter (>1µm) axons, they surround the axon, defasciculating it from the rest of the bundle in order to establish a 1:1 relationship between a single SC (now termed a ‘pro-myelinating’ SC) and a segment of that particular axon (Berti et al., 2011). Throughout this process immature SCs proliferate as needed to accommodate the number of axons and myelination is suppressed, presumably by the activity of negative regulators of myelination, including axon-SC Notch signalling, and the expression of transcription factors such as c-Jun, Pax3 (paired box 3), and Id2/4 (inhibitor of DNA binding 2/4) (Kioussi et al., 1995; Jessen and Mirsky, 2008; Parkinson et al., 2008; Woodhoo et al., 2009; Doddrell et al., 2012). In addition, a variety of molecules that control the process of radial sorting have now been identified, including laminin, laminin receptors, the Rho GTPases Rac and Cdc42 (cell division control protein 42), focal adhesion kinase, and leucine-rich, glioma activated 4 (reviewed in Jessen and Mirsky, 2008).

Following defasciculation pro-myelinating SCs promptly activate a molecular program to generate myelin sheaths (Svaren and Meijer, 2008; Pereira et al., 2012). Unlike oligodendrocytes in the CNS, each SC only myelinates a segment of a single axon (hence peripheral myelination occurs 1:1), and this is accomplished by wrapping around the axon repeatedly so that multiple layers of SC cytoplasmic membrane envelope those segments. Myelination requires the production of vast quantities of membrane lipids and myelin proteins including MAG, PMP-22 (peripheral myelin protein-22), MBP (myelin basic protein), and P₀ (protein zero), and the entire process is under the control of a variety of transcription factors that are known as positive regulators of myelination, such as: Sox10, Oct6 (octamer-binding transcription factor 6), Brn2 (Brain 2), NFATc4 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-4),
Yy1 (Yin yang 1), NF-kB, SREBPs (sterol regulatory element-binding proteins), and Krox-20 (aka: Egr2 [early growth response gene 2]) (reviewed in: Nagarajan et al., 2002; Svaren and Meijer, 2008; Pereira et al., 2012). Of these factors, Krox-20 plays the most central role, as this transcription factor is a primary target of all the other positive transcriptional regulators of myelination with the exception of SREBPs (Pereira et al., 2012); which are specifically involved in regulating cholesterol and fatty acid synthesis during myelination (Verheijen et al., 2009). In addition, Krox-20 acts as a master regulator for SC myelination, as this factor promotes cell cycle exit, activates numerous myelin genes including \textit{pmp22}, \textit{mpz} (myelin protein zero), and \textit{mbp}, suppresses the expression of myelination inhibitors such as \textit{pax3} and \textit{id2/4}, and is absolutely essential for SC myelination and myelin maintenance (Topilko et al., 1994; Decker et al., 2006; Jessen and Mirsky, 2008; Mirsky et al., 2008).

As radial sorting proceeds during neonatal development it results in the progressive erosion of axon bundles into smaller units (Webster and Favilla, 1984) and those axons with small diameters (<1\textmu m), which are destined to remain unmyelinated, are eventually ensheathed by non-myelinating SCs (Woodhoo and Sommer, 2008). Non-myelinating SCs typically ensheath more than one axon, each of which is normally accommodated in an individual mesaxon, which resembles a trough near the surface of the SC that is most often covered by SC processes (Griffin and Thompson, 2008). However, 1:1 ensheathment of axons by non-myelinating SCs is not uncommon in the PNS (Sharghi-Namini et al., 2006), as the number of axons ensheathed by a single cell differs among different nerves and in different regions along a single nerve, often approaching 1:1 as nerves approach their targets in the periphery (Murinson and Griffin, 2004; Griffin and Thompson, 2008).
These steps in SC lineage development are highly dependent on axon-derived NRG1 type III, which promotes proliferation of SCs to match axon numbers during radial sorting, but also plays a direct and essential role in controlling the transition from immature SCs to mature myelinating and non-myelinating phenotypes (Jessen and Mirsky, 2010). Although the decision to myelinate a particular axon appears to be determined by the physical diameter of the axon, it is actually determined by the amount of NRG1 type III expressed on each axon’s membrane (Nave and Salzer, 2006). Axons with high levels of NRG1 type III are ensheathed 1:1 by pro-myelinating SCs for subsequent myelination and those with low levels of NRG1 type III are ensheathed by non-myelinating SCs (Taveggia et al., 2005; Nave and Salzer, 2006). The amount of NRG1 type III on each axon also determines the thickness of the myelin sheath (Michailov et al., 2004), and a threshold level of NRG1 type III is even required to ensure normal ensheathment by non-myelinating SCs in Remak fibres (Taveggia et al., 2005; Fricker et al., 2009).

In addition to the influence of NRG1 type III and pro-myelinating transcription factors (e.g., Krox-20), a variety of other factors are known to play a role in SC proliferation, radial sorting, ensheathment, and myelination during the differentiation of mature SC phenotypes. Those factors include extracellular signals from axons (e.g., Notch signalling) and the extracellular matrix (ECM; e.g., laminins), cell adhesion molecules (e.g., L1 and integrins), downstream intracellular effectors such as Rho GTPases (e.g., Rac1 and Cdc42) that directly regulate SC cytoskeletal dynamics, secretases (e.g., BACE1 and TACE) that control the post-translational modification of NRG1, transcription factors that act as negative regulators of myelination (e.g., c-Jun and Pax3), and a variety of intracellular signalling pathways (e.g., PLCγ,
MEK and PI3K) and epigenetic molecules (e.g., HDACs and miRNAs) that directly influence myelin gene expression (Bradley and Jenkison, 1975; Stirling, 1975; Weinberg et al., 1975; Haney et al., 1999; Chen and Strickland, 2003; Yang et al., 2005; Mirsky et al., 2008; Parkinson et al., 2008; Pereira et al., 2010; Berti et al., 2011; Jacob et al., 2011; Doddrell et al., 2012; Pereira et al., 2012). A number of factors have also been identified that are required for the maintenance of myelin in adult peripheral nerve, including SC-expressed MAG (Schachner and Bartsch, 2000), Krox-20 (Decker et al., 2006) and Sox10 (Bremer et al., 2011), as well as the axonal expression of the prion protein PrPc (Bremer et al., 2010).

1.6.2 SC dedifferentiation

Unlike most other cell types in adult mammals, mature myelinating and non-myelinating SCs can readily dedifferentiate to take on a less mature, proliferative phenotype under appropriate conditions (Jessen and Mirsky, 2010). In vivo SC dedifferentiation is triggered by axonal damage and the loss of axon contact due to Wallerian degeneration (Mirsky et al., 2008). As the axons in the portion of peripheral nerve distal to the injury site degenerate, the SCs associated with those axons dedifferentiate to assume a less mature, growth-promoting, proliferative phenotype characterized by the up-regulation of regeneration-associated genes, including neuregulin receptors, p75, GFAP, GAP-43 (growth-associated protein-43), netrin-1 and key transcription factors including c-Jun, Pax3, Id2/4 (Fu and Gordon, 1997; Mirsky and Jessen, 1999; Mirsky et al., 2008; Parkinson et al., 2008; Doddrell et al., 2012; Fontana et al., 2012).

In myelinating SCs that process begins with the down-regulation of myelin-associated genes, which brings a stop to the production of myelin proteins within 48 hours of injury (Trapp
et al., 1988; White et al., 1989; Gupta et al., 1990; LeBlanc and Poduslo, 1990; Bosse et al., 1994). After their axons degenerate, the nucleus of each denervated SC moves to the centre of the internode and the myelin sheath splits on either side of the nucleus to release the SC, which begins to proliferate while the remaining myelin sheath is partitioned longitudinally to form small ovoids that are subsequently degraded (Stoll et al., 1989; Stoll and Hartung, 1992; Liu et al., 1995). Less is known about the process of dedifferentiation that occurs for non-myelinating SCs, but in contrast to their myelinating peers, which only dedifferentiate in response to injury to the axons they actually ensheath, non-myelinating SCs also dedifferentiate in response to the injury of nearby axons that they are not directly associated with and thus act as “first responders” following local axonal damage (Murinson et al., 2005). Dedifferentiated SCs play a number of roles in reshaping the environment of the distal nerve segment into one that is supportive of axonal regeneration, and thus the ability of SCs to dedifferentiate under appropriate conditions is a key factor in the success of peripheral nerve regeneration (see below).

Although the dedifferentiation of myelinating SCs begins with the downregulation of myelin genes, it is important to point out that the process of dedifferentiation is not merely the result of reduced activity in pro-myelinating signalling pathways. Indeed, dedifferentiation is known to involve the activation of specific receptors, intracellular signalling pathways, and transcription factors in a manner that is analogous to myelination, but with the opposite effect (Mirsky et al., 2008). For example, Notch signalling is elevated in injured nerves and appears to accelerate the rate of dedifferentiation, whereas transcription factors such as c-Jun, Pax3 and Sox2 are upregulated by SCs after injury and appear to play important roles in dedifferentiation by acting both as negative regulators of myelination and positive regulators of cell cycle re-entry
(Le et al., 2005; Mirsky et al., 2008; Parkinson et al., 2008; Doddrell et al., 2012). The in vivo influence of many of those factors remains speculative, but c-Jun has been shown to play an essential role in SC dedifferentiation following nerve injury in mice, as the absence of c-Jun specifically in SCs was recently demonstrated to cause the failure of SC dedifferentiation resulting in a dramatic impairment of peripheral nerve regeneration (Fontana et al., 2012).

As one might expect, the loss of axon contact following the isolation and purification of SCs from peripheral nerve also triggers SC dedifferentiation (Mirsky et al., 2008). As a result, the cultured N-SCs utilized in transplantation-based repair of the injured PNS and CNS (such as those used here in Chapter 3 and 4) actually represent a population of dedifferentiated SCs. This is an important point, particularly with regard to how those cells compare to SCs generated from sources other than peripheral nerve, such as SKP-SCs, as although dedifferentiated SCs upregulate their expression of many of the genes associated with the immature SC stage of development (Mirsky et al., 2008), those cells are not identical to immature SCs (Le et al., 2005; Jessen and Mirsky, 2008) and their re-differentiation into mature myelinating and non-myelinating SC phenotypes is similar, but not an exact match, to their developmental maturation (Pereira et al., 2012).

1.6.3 Sensory versus motor SC phenotypes

In addition to the development of myelinating and non-myelinating phenotypes, and the phenotypic differences associated with SCs at different stages of development / differentiation, SCs are known to display distinct sensory and motor phenotypes based on whether they are associated with sensory or motor nerves in vivo (Höke et al., 2006). Sensory and motor SCs differ in terms of their gene expression (Jesuraj et al., 2012), their migratory and proliferative
properties \textit{in vitro} (He et al., 2012), their growth factor production in intact and denervated peripheral nerve, and their ability to support the growth of sensory versus motor axons in the injured PNS (Höke et al., 2006). SCs are known to maintain their sensory or motor phenotype even after undergoing dedifferentiation and proliferation both \textit{in vitro} and \textit{in vivo}, and although those phenotypes change over time in culture and following inappropriate axonal reinnervation in the injured PNS (e.g., sensory axon reinnervation of motor SCs), they maintain distinct phenotype-specific properties even after 1 month \textit{in vitro} and retain some evidence of their original phenotype even after prolonged reinnervation with inappropriate axons (Höke et al., 2006; He et al., 2012; Jesuraj et al., 2012). To date, none of the axon-derived signals responsible for the specification of SCs to a sensory or motor phenotype have been identified, so the underlying mechanisms of that process remain a mystery.

1.6.4 Axon-derived signals control SC development, differentiation and dedifferentiation

From the SCP stage of development onwards SCs are continuously associated with axons and constantly exposed to axon-derived signals that are absolutely essential to the development of SCs in the PNS (Mirsky et al., 2008; Woodhoo and Sommer, 2008). Although a number of axon- and ECM-derived extracellular signals are known to play a role in SC development \textit{in vivo}, arguably the most important extracellular signal in SC lineage progression is axon-derived neuregulin-1 (NRG1) (Garratt et al., 2000; Nave and Salzer, 2006; Birchmeier and Nave, 2008; Mirsky et al., 2008; Jessen and Mirsky, 2010).

NRG1 signals to SCs via heterodimeric ErbB2/3 (erythroblastic leukemia viral oncogene homolog-2/3) tyrosine kinase receptors present on SCs at every developmental stage (Garratt et al., 2000; Nave and Salzer, 2006; Birchmeier and Nave, 2008). Although SCs will respond to
any NRG1 isoform that binds ErbB2/3, studies using mutant mice have demonstrated that the transmembrane NRG1 type III isoform, which is the most abundant NRG1 isoform in PNS axons, is also the most important NRG1 isoform for SC development in vivo, as NRG1 type III-ErbB signalling plays a critical role at every stage of SC development (Meyer and Birchmeier, 1995; Meyer et al., 1997; Birchmeier and Nave, 2008). For example, SCPs are critically dependent on axon-derived NRG1 type III for survival (Dong et al., 1995; Jessen and Mirsky, 2005) and that signal also appears to play a role in their migration and proliferation during embryonic nerve growth (Birchmeier and Nave, 2008). In addition, NRG1 type III promotes the proliferation of immature SCs to match axon numbers during radial sorting and the level of NRG1 type III expressed on the axonal membrane determines whether the SCs associated with that fibre will take on a non-myelinating or myelinating phenotype, and also regulates the thickness of the resulting myelin sheath in the latter case (Michailov et al., 2004; Taverggia et al., 2005; Nave and Salzer, 2006; Jessen and Mirsky, 2010). Soluble NRG1 isoforms are known to be potent stimulators of SC proliferation in vitro as well (Dong et al., 1995), and the combination of NRG1-β and low doses of forskolin (which potentiates NRG1-induced SC proliferation) was used to drive the differentiation of SKP-SCs and the expansion of SCs generated from both SKPs and nerve in the present work.

Beyond their role in SC lineage progression during development, axonal signals also play key roles in the specification of modality-specific SC phenotypes and the maintenance of SC phenotypes and SC myelin in both the intact and injured adult nerve. Unidentified axon-derived cues guide the specification of SCs to a sensory or motor phenotype during development and the sensory or motor modality of axons that reinnervate denervated SCs tends to drive those cells
towards the same SC phenotype following injury (Höke et al., 2006). Another example of the influence of axonal factors on SCs in adult nerve comes from the work of Bremer et al. (2010), who demonstrated that the expression of the prion protein PrP<sup>c</sup> on axonal membranes is essential for the maintenance of SC myelin in the intact adult PNS. Although mutant mice lacking axon-specific PrP<sup>c</sup> expression generate normal SC myelin during development, they develop a chronic demyelinating polyneuropathy the symptoms of which begin to appear immediately after the completion of peripheral myelination, at approximately 10 weeks of age (Bremer et al., 2010). It is interesting to note here, that the demyelination induced by PrP<sup>c</sup> ablation does not appear to occur as a result of SC dedifferentiation, as the expression of the SC myelin proteins CNPase, P<sub>0</sub>, MAG and PMP-22 remained unaffected.

Injury induced changes in axonal signalling are also thought to drive SC dedifferentiation and demyelination during Wallerian degeneration in the PNS. In addition to the central role of NRG1-ErbB signalling in guiding SC development, that signal may also help to initiate the SC response to axonal damage, as the activation of SC ErbB receptors occurs within minutes of peripheral nerve injury and appears to be sufficient to induce demyelination in myelinating SC-axon co-cultures (Guertin et al., 2005). These effects have not been linked to NRG1 type III, but have been shown to result from the addition of soluble NRG1 type II (a.k.a. glial growth factor [GGF]) to myelinating SC-DRG (dorsal root ganglion) co-cultures (Zanazzi et al., 2001); suggesting that different NRG1 isoforms may play opposing roles in SC differentiation and dedifferentiation. Other axon-derived signals that are thought to be involved in SC dedifferentiation and demyelination following nerve injury include Notch-signalling via axonal Jagged (Woodhoo et al., 2009; Pereira et al., 2012) and injury-induced expression of axonal
calcitonin gene-related peptide (CGRP) (Li et al., 2004; Toth et al., 2009). Furthermore, the activation of SC Toll-like receptors (TLRs) by axon-derived calpain (Bosse, 2012), the release of TLR ligands (e.g., heat shock proteins and DNA) due to axonal membrane disruption (Pineau and Lacroix, 2009), and/or injury-induced changes in the lipid composition of axonal membranes (Boivin et al., 2007) have all been speculated to play a role in activating the production of pro-inflammatory cytokines and chemokines in SCs following PNS injury, so those signals may also act to trigger SC dedifferentiation and demyelination in vivo. During and after regeneration in the PNS dedifferentiated SCs re-differentiate into myelinating or non-myelinating SCs based not on their original phenotype in the uninjured nerve, but again on signals from the axons with which they associate.

Thus, axon-derived signals appear to be critical, not only to the survival, migration, proliferation, and differentiation of SCs during PNS development, but also to the appropriate ensheathment/myelination of each axon, the maintenance/specification of mature SC phenotypes, the integrity of SC myelin sheaths throughout adulthood, and the phenotypic changes that occur in SCs in response to PNS injury (Jessen and Mirsky, 2005; Höke et al., 2006; Mirsky et al., 2008; Bremer et al., 2010; Bosse, 2012). As such, it seems clear that the phenotype of SCs is largely determined by the axons with which those cells associate, which bodes well for the use of exogenous SCs to treat SCI, as it suggests that SCs should be able to alter their phenotypes to suit the needs of injured or demyelinated axons they encounter in CNS.

1.6.5 SC functions during development and in adulthood in the intact PNS

While axonal signals are essential to the development and maintenance of the SC lineage, the opposite is also true, as SCs and their precursors are required for normal nerve development
and mature SCs are required to maintain long-term axon integrity and support normal nerve function in the adult PNS (Jessen and Mirsky, 2005; Nave and Trapp, 2008). In mutant mice that lack SCPs or in which those cells die prematurely, immature SCs fail to develop, normal peripheral nerve fasciculation fails to occur (Morris et al., 1999; Woldeyesus et al., 1999) and most sensory neurons in the DRG and motor neurons in the spinal cord die prior to birth, but only after reaching their target tissues (Riethmacher et al., 1997; Garratt et al., 2000; Britsch et al., 2001). Similarly, loss of mature myelinating (Trapp and Quarles, 1982; Garcia et al., 1998; Krajewski et al., 2000) and non-myelinating (Chen et al., 2003) SCs is also associated with axonal degeneration and neuronal death both postnatally and in adulthood. It is primarily believed that these effects are due to the fact that SCs and their precursors provide an important source of trophic support (e.g., glial-derived neurotrophic factor [GDNF]) for axons/neurons both during development and in adult tissues (Chen et al., 2003; Jessen and Mirsky, 2005), although back-signalling via interactions between membrane-bound receptors (e.g., NRG1 type III-ErbB2/3) may also provide the SC-derived signal that maintains axon integrity and neuronal survival (Riethmacher et al., 1997; Haney et al., 1999; Britsch et al., 2001; Nave and Salzer, 2006). SCPs and immature SCs also produce factors (e.g., desert hedgehog [DHH]) that are essential to the normal formation of protective nerve sheaths (particularly the perineurium and epineurium) during PNS development (Mirsky et al., 1999; Parmantier et al., 1999). Proper radial sorting by immature SCs is also required for normal nerve fasciculation and myelination, as abnormal fasciculation and hypomyelination are evident in mutant mice with defects that prevent radial sorting from occurring normally, including: Nfat1c, Rac1, β1 integrin, and laminin mouse mutants (Feltri et al., 2002; Chen and Strickland, 2003; Yang et al., 2005; Benninger et al., 2007; Nodari et al., 2007; Kao et al., 2009).
The main function of mature myelinating SCs is rather obvious, as myelination is well known to speed up axonal impulse conduction, but besides providing the fatty insulation that surrounds PNS axons, myelinating SCs also provide signals that guide the sequential assembly of multi-protein complexes, including cell adhesion molecules, ion channels, and scaffolding proteins, into distinct domains at and in the vicinity of the node of Ranvier, all of which are essential for establishing efficient saltatory conduction (Salzer et al., 2008). In addition, myelinating SCs are also known to profoundly influence axonal properties, including axon calibre, organelle content and rates of axonal transport even in adult nerve (de Waegh et al., 1992; Yin et al., 1998; Jessen and Mirsky, 2005); all of which are vulnerable in demyelinating diseases (Pereira et al., 2012). The functions of non-myelinating SCs in the nerve trunk have been studied far less than those of myelinating SCs, but in addition to their role in maintaining axon integrity and promoting neuron survival, proper ensheathment by these cells appears to be essential for normal axonal function and even the maintenance of axon integrity and neuronal survival at least some PNS neuronal populations (Haney et al., 1999; Chen et al., 2003; Nave and Trapp, 2008; Fricker et al., 2009). One of the axon populations ensheathed by non-myelinating SCs are the small diameter C-fibres that play an extremely important role in pain sensation, and it is thought that even minor deviations in ensheathment of those axons may cause abnormalities in nociception (Campana, 2007).

1.6.6 The role of SCs in spontaneous repair and recovery after PNS injury

In sharp contrast to the situation in the CNS, spontaneous axonal regeneration is the norm following injury to the adult mammalian PNS. SCs have long been known to play a key role in
that process, and are essential to the efficient PNS regeneration that is generally required to ensure successful reinnervation and functional recovery (Gaudet et al., 2011; Bosse, 2012).

SCs are the first cells to respond to injury in the peripheral nerves, and these cells are known to alter to their gene expression within hours of injury and long before axon degeneration actually occurs (Liu et al., 1995; Shamash et al., 2002; Guertin et al., 2005; Murinson et al., 2005). Indeed, SCs have been characterized as ‘sentinels’ in the PNS, analogous to microglia in the CNS, as they possess a number of characteristics that allow them to play important roles in the early immune/inflammatory response to PNS injury (Goethals et al., 2010). For example, SCs constitutively express a variety of TLRs and at least one purinergic receptor (P2X$_7$) that allow them to respond to endogenous ligands that are not normally present in the extracellular space (e.g., heat shock proteins, RNA, DNA, degraded ECM, and ATP) (Grafe et al., 1999; Boivin et al., 2007; Pineau and Lacroix, 2009; Goethals et al., 2010), and they produce pro-inflammatory cytokines/chemokines including TNF$\alpha$, iNOS, IL-1$\alpha$/β (interleukin-1$\alpha$ and -1$\beta$), IL-6 (interleukin-6), LIF (leukemia inhibitory factor) and MCP-1 (monocyte chemoattractant protein-1) that contribute to the recruitment and accumulation of hematogenous macrophages (Stewart et al., 1995; Siebert et al., 2000; Shamash et al., 2002; Tofaris et al., 2002; Karanth et al., 2006; Lee et al., 2006; Chattopadhyay et al., 2007); which is an essential step in successful PNS regeneration (Gaudet et al., 2011; Bosse, 2012). SCs also express major histocompatibility complex (MHC) class II molecules, which enables them to act as antigen presenting cells, although it remains unclear whether they perform that function \textit{in vivo} (Holtzman and Novikoff, 1965; Wekerle et al., 1986; Hirata et al., 1999; Meyer zu Horste et al., 2008). Together those findings suggest an important role for SCs in the inflammatory response after PNS injury and
particularly in initiating the cascade of cytokine and chemokine production that draws hematogenous macrophages into the injured nerve (Shamash et al., 2002; Gaudet et al., 2011).

In addition to helping to recruit hematogenous macrophages to the site of PNS injury, dedifferentiated SCs take on a phagocytic phenotype and engulf extracellular debris, including the remnants of their own myelin sheaths, which undergo enzymatic digestion within the SC cytoplasm (Stoll et al., 1989; Fernandez-Valle et al., 1995; Hirata and Kawabuchi, 2002; Koeppen, 2004). Although endoneurial macrophages that reside in the PNS also contribute to myelin debris clearance, dedifferentiated/denervated SCs are the major phagocytic cell found in the distal nerve segment for the first five days after injury, as large numbers of blood-borne macrophages do not appear in the nerve until 5-7 days post-injury (Liu et al., 1995; Perry et al., 1995; Mueller et al., 2003; Omura et al., 2005). Despite their later arrival, the blood-borne macrophages are more effective at removing myelin debris than the SCs or macrophages that reside in the nerve, and those hematogenous cells play a key role in the rapid clearance of myelin debris that is required for efficient PNS axonal regeneration (Dailey et al., 1998; Stoll and Muller, 1999; Shen et al., 2000; Barrette et al., 2008). In chronically denervated rat and rabbit tibial nerves, myelin removal in the distal nerve segment is largely complete by 7 weeks post-injury (Weinberg and Spencer, 1978), and the removal of myelin-associated inhibitors of axonal growth from the distal nerve paves the way for successful PNS regeneration (Gaudet et al., 2011).

The proliferation of dedifferentiated SCs begins around two days post-injury and peaks approximately 2 days later in the rat (Pellegrino et al., 1986; Liu et al., 1995; Murinson et al., 2005). Where the basal lamina remains intact following injury, proliferating SCs are confined to
that structure and those SCs align to form longitudinal chains of cells known as bands of Büngner, which extend through the distal nerve segment to populate the basal lamina tube from the site of injury all the way to distal targets (Stoll et al., 1989; Fawcett and Keynes, 1990; Liu et al., 1995; Stoll and Muller, 1999). In addition to forming bands of Büngner within their intact basal lamina, SCs are also known to extend similar processes from severed nerve ends in advance of regenerating axons (Reynolds and Woolf, 1992; Son and Thompson, 1995a), and both of those structures act as growth-supportive substrates that help guide and promote the regeneration of axons back to their denervated targets (Bunge et al., 1989; Stoll et al., 1989; Son and Thompson, 1995a, b; Fu and Gordon, 1997). Importantly, the existence of bands of Büngner has been confirmed in injured human peripheral nerves, and similar to the findings in rodents, regenerating axons were always found to associate with those structures in the injured human PNS (Terenghi et al., 1998). Successful regeneration can occur even if the formation of those structures is blocked (i.e., by blocking SC proliferation), provided that the basal lamina remains intact (Yang et al., 2008). However, when injury causes a loss of continuity in the basal lamina, such as that occurring after transection, the proliferation of SCs and their migration from nerve stumps are critical for the directional outgrowth of axons from the proximal stump of the nerve (Chen et al., 2005; Webber and Zochodne, 2010).

In addition to providing a supportive substrate for axonal regeneration, SCs also up-regulate the production of a wide variety of substances, including ECM components, cell surface adhesion molecules, growth factors, neurotrophins, cytokines/chemokines, and axon guidance molecules following dedifferentiation, many of which have been shown to facilitate neuronal survival, axonal regeneration, or collateral sprouting either in vitro or in vivo following PNS
injury (Webber and Zochodne, 2010). The SCs that form processes and bands of Büngner elaborate a scaffold of basement membrane proteins including axonal growth-promoting isoforms of laminin, fibronectin, tenascin, and collagen (Martini, 1994; Zhang et al., 1995a; Fu and Gordon, 1997; Vogelezang et al., 1999; Gardiner et al., 2007; Gardiner, 2011), and those cells are known to up-regulate their expression of regeneration-promoting cell surface adhesion molecules, including: NCAM (neural cell adhesion molecule), L1, L2 (HNK-1) and NCad (neural cadherin) (Martini, 1994; Stewart et al., 1995; Zhang et al., 1995b; Fu and Gordon, 1997; Chen et al., 2007).

Denervated SCs also produce an impressive array of neurotrophic factors and neuropoeitic cytokines, including: NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT (neurotrophin)-3 and -4, IGF, CNTF (cilliary neurotrophic factor), GDNF, LIF, FGF-2 (fibroblast growth factor 2), IL-6, artemin and pleiotrophin (Heumann et al., 1987; Acheson et al., 1991; Friedman et al., 1992; Meyer et al., 1992; Curtis et al., 1994; Springer et al., 1995; Hammarberg et al., 1998; Fansa et al., 1999; Grothe et al., 2000; Höke et al., 2002; Höke et al., 2006; Gordon, 2009; Fontana et al., 2012; Patodia and Raivich, 2012); which may not only promote neuronal survival and axonal growth, but may also act as diffusible guidance cues during regeneration (Patodia and Raivich, 2012). SCs in the distal nerve are also known to alter their expression of cell-surface guidance molecules following injury. For example, at the regenerating nerve front those cells up-regulate their expression of the growth-cone-attracting netrin-1 receptor DCC (deleted in colorectal cancer) and down-regulate their expression of growth-cone-repelling netrin-1 receptor Unc5H2 (uncoordinated 5H2) (Webber et al., 2011). Recent evidence also indicates that regenerating axons receive polyribosomes from
SCs, which suggests that SCs may support local axonal protein synthesis directly by transferring protein synthetic machinery and mRNA to their associated axons (Court et al., 2008; Court et al., 2011).

The relevance of many of those factors to PNS regeneration in vivo has yet to be demonstrated, but even for those factors that are known to play a role in vivo (e.g., laminin-2/-8, GDNF, artemin, IL-6, and DCC-netrin signalling; (Zhong et al., 1999; Chen and Strickland, 2003; Parrinello et al., 2010; Fontana et al., 2012), the relative importance of each to the success of PNS regeneration and functional reinnervation varies according to the axons in question, as the PNS is composed of a heterogeneous collection of neurons that give rise to a variety of axon populations that each express specific sets of receptors and thus respond to different growth-related environmental cues (Patodia and Raivich, 2012). For example, substantial heterogeneity exists in the expression of specific integrin receptors in different axon populations, and dictates differing ECM-responsiveness post-injury (Guan et al., 2003), which is complicated even further by the fact that subpopulations of neurons respond differentially to ECM components (e.g., laminin) as a function of growth factor receptor activation (Tucker et al., 2006; Paveliev et al., 2007). Furthermore, the dedifferentiated state of all SCs is not equal, as sensory SCs show enhanced proliferation and migration compared to motor SCs in vitro (He et al., 2012) and those phenotypes also differ in terms of their expression of regeneration-associated genes and growth factors both in vitro and in the injured peripheral nerve (Höke et al., 2006; Jesuraj et al., 2012). Thus, the ability of SCs to promote axonal growth varies not only according the specific axon populations that were injured, but also according to the population of SCs available to respond to that injury.
Besides providing a favourable substrate, trophic support, and guidance for regenerating axons, dedifferentiated SCs are known to re-differentiate upon re-establishment of axon contact, which triggers the return of normal gene expression and ensheathment or myelination of regenerating axons by mature myelinating and non-myelinating SC, respectively (Fawcett and Keynes, 1990; LeBlanc and Poduslo, 1990; Stoll and Muller, 1999; Mirsky et al., 2008). Indeed, re-differentiation occurs rapidly enough that SCs containing myelin debris can occasionally be found remyelinating regenerating PNS fibres in vivo (Koeppen, 2004); a rather poignant demonstration of the phenotypic plasticity of SCs. Re-differentiation is the final stage of successful PNS regeneration, as the return of appropriate SC ensheathment and myelination is required for the maintenance of axonal health and the full return of normal neurological functions.

1.6.7 Therapeutic application of SCs following PNS injury

Although spontaneous axonal regeneration is relatively robust in the PNS, that process is not perfect, and regenerative success (defined as the reinnervation of target structures and full functional recovery) is by no means guaranteed (Höke, 2006). The outcome of peripheral nerve injury is particularly poor when injury occurs far from the nerve’s target tissue, causing prolonged denervation of the distal nerve segment, or in the case of large gap injuries (Fu and Gordon, 1995a; Hood et al., 2009). In those situations, spontaneous regeneration is frequently incomplete, misdirected or associated with debilitating neuropathic pain (Sunderland, 1978). The further proximal a lesion occurs, the longer the distance that regenerating axons must travel to reach target tissues, and this can leave the distal stump denervated for long periods of time, particularly in humans, where the rate of nerve regeneration is only about 1mm/day (Sunderland,
1947). To make matters worse, surgical interventions in cases where spontaneous regeneration fails are typically delayed to assess the extent of spontaneous recovery, and this prolongs the period of denervation in the distal nerve segment. In light of these issues, virtually every severe human nerve injury involves chronic denervation of the distal nerve segment (Fu and Gordon, 1997).

The growth-supportive environment that develops in the distal nerve segment following Wallerian degeneration, due in part to the properties and behaviours of dedifferentiated SCs, is not maintained unless axonal contact is re-established in a timely fashion (Weinberg and Spencer, 1978; Pellegrino and Spencer, 1985; Taniuchi et al., 1988). Prolonged denervation leads to diminished regeneration over time and can eventually result in the complete failure of that process, and this is thought to at least partly result from dramatic changes that occur in SCs when they are deprived of axonal contact for too long (Fu and Gordon, 1995a). Over time post-injury, SCs gradually down-regulate their production of neurotrophic factors and receptors, ErbB neuregulin receptors and cell adhesion molecules, and this process results in a progressive decline in SC proliferation, migratory potential, and ability to support axonal regeneration, eventually leading to SC atrophy (Fu and Gordon, 1997; Li et al., 1997; You et al., 1997; Hall, 1999; Gordon, 2009). Without re-establishment of axonal contact, SCs only retain their full growth supportive capacity in the distal nerve for 1-2 months (Li et al., 1997; You et al., 1997; Sulaiman and Gordon, 2000), and by several months after denervation many of those cells are lost to apoptosis (Pellegrino et al., 1986; Sulaiman et al., 2002; Ebenezer et al., 2007). In the absence of viable SCs the basal lamina eventually fragments and disappears as well (Pellegrino et al., 1986; Griffin and Thompson, 2008), after which successful spontaneous regeneration is
practically impossible. It is of interest to note, that SCs that survive chronic denervation retain the capacity to remyelinate any axons that do manage to regenerate (Sulaiman and Gordon, 2000). Prolonged denervation is also well-correlated with poor functional outcome following surgical repair (Fu and Gordon, 1995b; Sulaiman et al., 2002; Aydin et al., 2004).

The type of injury also influences the rate and thus the likelihood of success in PNS regeneration. Following crush injury, most of the basal lamina remains intact, and both axons and SCs can extend/migrate along that scaffold, whereas following transections the continuity of the basal lamina is completely lost, so no scaffold exists to expedite the growth and guidance of axons back to their original targets (Tomita et al., 2009). As a result, the average rate of regeneration varies depending on the type of injury, reaching speeds of 3-4 mm/day for crush injuries but only 2-3 mm/day following transection in rodents (Stoll and Muller, 1999). Those values are assuming the presence of SCs that are capable of responding to injury, and the rates of regeneration and likelihood of regenerative success are greatly reduced in the absence of those cells. Indeed, the presence of viable SCs is considered a prerequisite for axonal regeneration across long peripheral nerve defects, particularly if those defects include nerve gaps (Fansa et al., 1999; Fansa and Keilhoff, 2004; Hess et al., 2007).

For gap injuries that are not amenable to direct ‘end-to-end’ nerve repair and when spontaneous peripheral nerve regeneration fails, the gold standard for the repair of PNS defects is the autologous nerve graft (Evans, 2000; Whitlock et al., 2009). In order to limit the resulting functional deficits, those grafts are typically harvested from ‘less critical’ sensory nerves, such as the sural nerve in humans (Hood et al., 2009), but in light of the fact that harvesting that nerve causes sensory deficits and may induce neuropathic pain (see below), a variety of alternative
materials, both natural (e.g., vein, muscle, collagen) and synthetic (e.g., silicone, polyglycolic acid), have been tested as potential conduits for long distance PNS regeneration (reviewed in: Evans, 2000; Meek and Coert, 2002; Wolford and Stevao, 2003). In the presence of SCs, nerve grafts are able to support substantial regeneration across gaps in peripheral nerve up to 6cm in length, but in the absence of SCs, as in acellular nerve grafts or muscle / vein conduits, neuroregenerative potential is generally limited to <3cm (Tang et al., 1993; Doolabh et al., 1996; Strauch et al., 1996; Hess et al., 2007). Those limitations are primarily thought to be due to the lack of trophic support normally provided by SCs (Strauch et al., 1996; Evans et al., 1998; Fansa et al., 1999; Boyd and Gordon, 2003; Hess et al., 2007). Evidence in support of that notion comes from the fact that the addition of SCs to acellular grafts or conduits is well known to facilitate regeneration over greater distances and similar effects have been noted with the addition of exogenous growth factors (Levi et al., 1994; Ansselin et al., 1997; Boyd and Gordon, 2003; Hess et al., 2007; Whitlock et al., 2010). Indeed, the addition of SCs to acellular grafts and artificial conduits is a common approach used in PNS injury treatment research, and SKP-SCs have previously been used with some success for precisely that purpose (Walsh et al., 2009).

1.6.8   Endogenous SCs in the CNS

1.6.8.1   The source of endogenous SCs in the injured CNS

Endogenous SCs have long been known to spontaneously remyelinate axons in the demyelinated CNS, both in animal models of demyelination and in humans afflicted with demyelinating disorders such as multiple sclerosis (MS) (Feigin and Ogata, 1971; Blakemore, 1975; Snyder et al., 1975; Itoyama et al., 1983; Itoyama et al., 1985; Yamamoto et al., 1991; Felts et al., 2005). Spontaneous SC remyelination in the CNS preferentially occurs in regions
devoid of astrocytes and is more extensive when lesions occur near potential sources of peripheral SCs, such as spinal/cranial roots, meningeal nerves or autonomic nerves associated with blood vessels (Snyder et al., 1975; Franklin and Blakemore, 1993; Duncan and Hoffman, 1997). In light of that evidence, it has generally been assumed that those cells originate from SCs in the PNS and simply invade the CNS in regions where the astrocytic glial limitans is disrupted by injury/disease. However, based on evidence that purified CNS stem/progenitor cells can generate SCs *in vitro* and following transplantation into regions of focal demyelination in the CNS (Mujtaba et al., 1998; Keirstead et al., 1999b; Chandran et al., 2004), and the fact that SCs show very limited migration through intact CNS parenchyma, it has also been suggested that those endogenous SCs may arise directly from resident CNS stem/progenitor cells (Blakemore, 2005). The latter hypothesis was recently confirmed in fate mapping experiments using the Cre-lox system, which demonstrated that endogenous *Pdgfra*+ and *Olig2*+ CNS progenitors give rise to most of the endogenous SCs found remyelinating axons in regions of chemically-induced focal demyelination in the CNS (Zawadzka et al., 2010). Thus it would seem that the endogenous SCs found in the CNS can originate from either the PNS or the CNS, and the latter appears to act as the primary source of those cells under certain circumstances.

Endogenous SCs have also been found in the CNS following traumatic SCI in a variety of mammals, including rodents (Bunge et al., 1994; Beattie et al., 1997; Brook et al., 1998; Brook et al., 2000; Totoiu and Keirstead, 2005), cats (Harrison and McDonald, 1977; Blight and Young, 1989), monkeys (Bresnahan, 1978) and humans (Feigin and Ogata, 1971; Kakulas, 1984; Bunge et al., 1993; Bruce et al., 2000; Norenberg et al., 2004; Guest et al., 2005). In humans the appearance of SCs in injured spinal cord tends to occur via two very distinct patterns, referred to
as schwannosis and atypical schwannosis (Guest et al., 2005). Schwannosis is characterized by the aberrant invasion of the CNS by highly fasciculated bundles of SCs and axons that arise directly from the injured nerve roots (Bruce et al., 2000; Norenberg et al., 2004; Guest et al., 2005), and is most common in severe SCI, particularly involving penetrating injuries, maceration of the spinal cord, or extensive damage to the glial limitans near spinal nerve roots (Blight and Young, 1989; Schwab and Bartholdi, 1996; Norenberg et al., 2004). In contrast, atypical schwannosis refers to the presence of endogenous SCs alone in the CNS parenchyma, which is more similar to the pattern observed in demyelinated lesions of the CNS, as those cells are found myelinating CNS axons in regions of the host parenchyma that are relatively devoid of astrocytes (Bresnahan, 1978; Bunge et al., 1994; Beattie et al., 1997). In cases of SCI involving schwannosis the endogenous SCs found in the injured CNS appear to originate directly from the periphery. However, in cases of SCI involving atypical schwannosis, the source of the endogenous SCs has yet to be definitively determined, and although migration from the periphery seems likely when injuries directly disrupt the glial limitans (Oudega and Xu, 2006), the findings of (Zawadzka et al., 2010) suggest that at least some of the endogenous SCs found in the spinal cord after traumatic injury may arise from CNS precursors, particularly following closed injuries that cause significant secondary demyelination (e.g., contusion or compression SCI).

1.6.8.2 The role in endogenous SCs in spontaneous CNS repair

In animal models of SCI endogenous SCs can be detected in the CNS during the first week post-injury (Beattie et al., 1997; Brook et al., 1998; Brook et al., 2000), whereas they are generally not detected until weeks or even months later in human cadaveric tissue (Kakulas,
Although delayed somewhat, the appearance of endogenous SCs after SCI in humans is very common, and has been reported in 82-90% of cadaveric samples from patients who survived >4 months after injury (Bruce et al., 2000; Norenberg et al., 2004). Furthermore, those cells are known to persist long-term in the injured spinal cord, as they have been found years or even decades after SCI in human cadaveric tissue (Kakulas, 1984; Bunge et al., 1993; Bruce et al., 2000; Guest et al., 2005; Buss et al., 2007). Whether or not those cells contribute to spontaneous repair and recovery in the injured CNS appears to primarily depend on whether they are associated with schwannosis or atypical schwannosis.

In cases of schwannosis, the invading SCs and axons from the periphery are typically walled off from the CNS by a dense glial scar and thus fail to integrate with spared CNS tissue and instead tend to form structures resembling peripheral neuromas (see below) within the injured spinal cord (Bruce et al., 2000; Norenberg et al., 2004; Guest et al., 2005; Buss et al., 2007). As a result, schwannosis is not generally associated with CNS repair or functional recovery, and to the contrary, it has actually been speculated to limit the regeneration of CNS axons and contribute to pain and spasticity in patients with chronic SCI (Bruce et al., 2000; Norenberg et al., 2004). Conversely, in cases of atypical schwannosis, endogenous SCs are often found remyelinating CNS axons in the tissue immediately adjacent to the lesion cavity, and those cells are sometimes found in large numbers, particularly following contusive injuries that involve a substantial degree of secondary demyelination in those regions (Blight and Young, 1989; Beattie et al., 1997; Guest et al., 2005). In animal models of MS and SCI, spontaneous remyelination of CNS axons by endogenous SCs has been associated with improved
electrophysiological functioning, including the return of saltatory conduction and near-normal conduction properties, and has even been correlated with some recovery of locomotor function (Blight and Young, 1989) (Pender, 1989; Felts and Smith, 1992; Jeffery and Blakemore, 1997; Jasmin et al., 2000; Murray et al., 2001; James et al., 2011). In addition, there is evidence suggesting that the endogenous SCs residing in the lesion site can promote the growth of some CNS axons into contusive/compressive cavities after SCI in rodents (Beattie et al., 1997; Brook et al., 1998), and the fact that SCs have been found myelinating axons that appear to be of CNS origin years or even decades after SCI or the onset of MS (Itoyama et al., 1983; Itoyama et al., 1985; Guest et al., 2005) indicates that endogenous SCs are capable of preserving the integrity and enhancing the survival of at least some CNS axon populations.

Thus, the evidence from animal models suggests that in the absence of peripheral nerve invasion (i.e., schwannosis), the response of endogenous SCs is not only a common component of the normal endogenous cellular response to SCI, but may also contribute to what little spontaneous recovery occurs in the injured CNS (Beattie et al., 1997). However, given that damage and functional deficits persist following SCI, the contribution of endogenous SCs to spontaneous repair is clearly insufficient to reverse the negative sequelae of SCI (Oudega and Xu, 2006).

1.6.9 Therapeutic applications of SCs in CNS injury and disease

The study of SCs as a potential treatment for CNS injury and disease began in earnest over 30 years ago, when Aguayo and colleagues definitively demonstrated that peripheral nerve (PN) grafts support the growth of CNS axons (Richardson et al., 1980). That work prompted a number of studies examining the ability of PN grafts to support the regeneration of various CNS
axon populations (see below). Meanwhile, growing interest in tissue culture and in vitro experimentation brought about the development of techniques to harvest and purify SCs from peripheral nerve, which provided a means to generate purified populations of N-SCs in culture for the first time, and prompted Richard Bunge (Bunge, 1975) to propose the application of those cultured cells for CNS repair (reviewed in Wiliams and Bunge, 2012). Towards that goal, new techniques were developed to increase the purity of N-SCs generated from the PNS (Wood, 1976; Brockes et al., 1979). Those developments paved the way for work examining the transplantation of suspensions of cultured N-SCs into the demyelinated CNS, which began around the same time that Aguayo and colleagues initiated their peripheral nerve graft experiments (Duncan et al., 1981), and also prompted studies examining the ability of cultured N-SCs to promote axonal growth when introduced into the injured CNS. Subsequent work optimizing the yield of N-SCs and their expansion in vitro facilitated the mass-production of those cells from rodent and human adult peripheral nerve for therapeutic applications (Morrissey et al., 1991; Levi et al., 1995a; Casella et al., 1996), and thus facilitated large-scale studies of N-SC transplantation in rodent injury models of SCI and demyelinating disease and improved the feasibility of their potential clinical application.

In light of the key role of endogenous SCs in spontaneous recovery following peripheral nerve injury and the fact that those cells are able to myelinate CNS axons as well as promote their growth, it comes as little surprise that SCs harvested from peripheral nerve (N-SCs) have garnered considerable attention as a potential exogenous cellular therapy for CNS repair. Indeed, those cells have long been studied as a potential treatment for both SCI and MS (Felts and Smith, 1992; Zujovic et al., 2007; Tetzlaff et al., 2011; Wiliams and Bunge, 2012), and were not only a
key component of the first treatment (PN grafts) to convincingly demonstrate that injured CNS axons could regenerate (Richardson et al., 1980; Berry et al., 1988; Smith and Stevenson, 1988), but were also the first cell type to be purified for transplantation into the demyelinated CNS (Duncan et al., 1981). The behaviour and reparative benefits of N-SCs transplanted into the injured/diseased CNS have largely been studied using four approaches: the grafting of peripheral nerves to repair CNS defects, the transplantation of cultured N-SCs into the intact and demyelinated CNS, the application of channels/conduits containing N-SCs to the transected spinal cord, and direct intraparenchymal transplantation of N-SCs following SCI (e.g., contusion, compression, or crush injuries). As a result of those efforts N-SCs are one of the most studied cell types in preclinical SCI and MS treatment research today (Zujovic et al., 2007; Tetzlaff et al., 2011), and those cells were not only chosen for the first clinical trial of a cellular therapy to treat MS (Halfpenny et al., 2002; Zujovic et al., 2007), but are currently the focus of multiple clinical trials in SCI (Saberi et al., 2011; Talan, 2012).

1.6.9.1 Peripheral nerve grafts applied to the CNS

The first definitive demonstration that injured CNS axons are able to regenerate into PN grafts came at the hands of Aguayo and colleagues, who bridged the fully transected spinal cord of the rat with an autologous sciatic nerve graft and used retrograde axonal tracing to demonstrate that axons originating in the CNS traversed the graft (Richardson et al., 1980). Subsequent work established that PN grafts readily support the ingrowth of nearby sensory and propriospinal axons, but only support the regeneration of long ascending or descending spinal axons when applied to injury sites near the cell bodies of those fibres (David and Aguayo, 1981; Richardson et al., 1982; Richardson et al., 1984; Oudega et al., 1994). For example, it was found
that supraspinal axon populations (e.g., CST and RST) generally failed to regenerate into PN
grafts applied at the thoracic or lumbar levels and ascending sensory fibres from the lumbar
DRG rarely regenerate into high cervical grafts (Richardson et al., 1982; Richardson et al., 1984;
Houle, 1991). In addition, regardless of their source (i.e., propriospinal, sensory, or supraspinal),
the axons that did enter PN grafts often failed to reach the distal graft-host interface, let alone
cross that boundary to enter the intact distal parenchyma (Richardson et al., 1982; Oudega et al.,
1994; Oudega and Hagg, 1996). Thus, on their own PN grafts applied to the injured spinal cord
were found to support limited supraspinal axon growth and more robust propriospinal and
sensory regeneration, but largely failed to promote the restoration of functional connections
across the lesion site following SCI.

In addition to demonstrating that PN grafts could support the growth of CNS axons, that
work also provided multiple demonstrations of SCs in peripheral nerve grafts ensheathing and
myelinating CNS axons (Richardson et al., 1980; David and Aguayo, 1981). Those cells were
also occasionally found ensheathing axons that had regenerated into distal host tissue (David and
Aguayo, 1981), although the latter was a relatively rare event, as the SCs from the graft were not
generally known to migrate into host CNS tissue (Fishman et al., 1983). In light of those
observations, and the appearance of astrocytes at the graft-host interface, it was speculated that
the astrocyte response to the graft was responsible for preventing graft-derived SCs from
entering the CNS environment (Fishman et al., 1983), and that notion was generally supported
by subsequent work transplanting segments of sciatic nerve into the intact and demyelinated
CNS (Sims et al., 1999).
Given the fact that SCs are the primary cellular component of those grafts, and in light of their role in spontaneous regeneration in the PNS, it was always assumed that those cells were primarily responsible for the positive effects associated with peripheral nerve repair of CNS defects. Confirmation of that hypothesis was eventually provided by experiments that demonstrated that acellular nerve grafts (generated by freezing and thawing the graft to kill the resident SCs), failed to support significant CNS axonal growth despite the continued presence of the growth supportive SC basal lamina (Berry et al., 1988; Smith and Stevenson, 1988). In addition to demonstrating that SCs are necessary for CNS regeneration into peripheral nerve grafts that work also indicated that those cells do more than just provide a supportive substrate for axonal growth.

1.6.9.2 N-SC transplantation into the intact and demyelinated CNS

The interest in N-SCs as a potential treatment for demyelinating disorders of the CNS was largely prompted by evidence indicating that SCs and SC myelin are resistant to MS pathology; such as the fact that demyelination of the PNS is rare in MS, the finding that endogenous SCs can spontaneous remyelinate the CNS of MS patients, and reports that those myelinating SCs are even occasionally found in MS plaques with ongoing loss of central myelin (Feigin and Ogata, 1971; Itoyama et al., 1983; Itoyama et al., 1985; Yamamoto et al., 1991; Felts and Smith, 1996). In light of that interest, much of the early work examining the basic biology of SC transplantation came from studies of N-SCs transplanted into animal models of CNS demyelination (reviewed in Duncan and Milward, 1995).

To date N-SCs have been extensively tested in a variety of models of demyelinating disease, including toxin-induced focal demyelination (e.g., lysolecithin or ethidium bromide) or
global demyelination resulting from experimental autoimmune encephalomyelitis (EAE) (Blakemore and Crang, 1985; Honmou et al., 1996; Iwashita and Blakemore, 2000; Shields et al., 2000; Kohama et al., 2001; Bachelin et al., 2005; Zujovic et al., 2012). That work has firmly established the ability of transplanted N-SCs (generated from rodent, macaque and human nerve) to remyelinate axons in the demyelinated rodent CNS and has also demonstrated that N-SC remyelination is associated with the restoration of axonal conduction (Honmou et al., 1996; Imaizumi et al., 2000; Kohama et al., 2001) and improved locomotor function (Bachelin et al., 2005) in models of focal demyelination, as well as significant recovery from clinical symptoms and decreased mortality following immune-mediated demyelination (Zujovic et al., 2012).

In addition to the ability to remyelinate denuded CNS axons, the ability of transplanted cells to survive and migrate through the intact CNS to reach regions of demyelination is generally thought to be a prerequisite for effective remyelination strategies (Woodhoo et al., 2007; Zujovic et al., 2007). In light of that prerequisite, N-SCs have been rather thoroughly studied following transplantation into the intact CNS. Some of those studies have shown that transplanted N-SCs are capable of limited migration through intact CNS tissue, particularly along blood vessels, the meninges, and the ependymal wall (Baron-Van Evercooren et al., 1993; Raisman et al., 1993; Baron-Van Evercooren et al., 1996). However, the majority of that work indicates that N-SCs largely fail to migrate through or integrate with intact astrocyte-rich host tissue, as those cells tend to remain at the site of injection, apparently walled off from the rest of the CNS by the astrocytic glial scar, and in the absence of denuded axons at that location they typically die within weeks of transplantation (Blakemore et al., 1986; Iwashita and Blakemore, 2000; Iwashita et al., 2000; Shields et al., 2000; Santos-Silva et al., 2007; Woodhoo et al., 2007).
The exception to this rule appears to be N-SC transplantation following diffuse inflammatory demyelination induced by EAE, as in that model N-SCs have been found to migrate away from the site of injection and mix with host astrocytes in the absence of a noticeable glial scar, and despite the fact that EAE is generally associated with astrogliosis and glial scar formation (Zujovic et al., 2012). The lack of migration and survival of N-SCs delivered to intact CNS parenchyma is thought to represent a significant limitation to their therapeutic potential in MS (Zujovic et al., 2007). In that regard, it is of interest to note that SCPs do not appear to suffer the same limitations, as those less mature SCs survive well following injection into intact tissue, migrate through normal CNS tissue to occupy sites of demyelination, and integrate well with host astrocytes (Woodhoo et al., 2007).

1.6.9.3 Bridges containing N-SCs applied to SCI transection models

Recognition of the central role of SCs in promoting the growth of CNS axons into nerve grafts and the development of techniques to harvest and purify N-SCs in vitro prompted a considerable amount of research examining the ability of transplanted N-SC suspensions to facilitate axonal regeneration after SCI, which was largely conducted by Richard and Mary Bunge at the Miami Project to Cure Paralysis (reviewed in: Oudega and Xu, 2006; Fortun et al., 2009; Bunge and Wood, 2012; Wiliams and Bunge, 2012). Much of the early work examining the transplantation of cultured N-SCs after SCI was conducted using full/partial transection models and acute transplantation paradigms and focused almost exclusively on histological analyses of axonal regeneration and the remyelination of regenerating fibres rather than behavioural outcomes. That work typically involved the removal of whole or partial segments of the spinal cord and the immediate application of synthetic guidance channels made of
polyacrylonitrile / polyvinylchloride (PAN/PVC) filled with N-SCs suspended in matrigel to bridge the resulting gap (Xu et al., 1995b; Xu et al., 1995a; Chen et al., 1996; Xu et al., 1997; Xu et al., 1999; Pinzon et al., 2001). Although those injury/treatment paradigms are not the most clinically relevant approaches, they allow for the unambiguous identification of regenerating fibres due to the complete absence of spared fibres within the graft/channel, and thus provide an ideal means for assessing the ability of SCs to promote CNS regeneration, similar to PN grafts.

By and large, those studies confirmed the results of work transplanting PN grafts into the injured spinal cord, as N-SCs were observed myelinating regenerating CNS axons within those bridges, and although the N-SC-seeded conduits were able to stimulate the growth of CNS axons, the fibres that responded were primarily of sensory and propriospinal rather than supraspinal origins, and regenerating axons largely failed to exit the conduit and thus did not re-enter the CNS parenchyma to form new synapses with spared host tissue (Xu et al., 1995b; Xu et al., 1995a; Chen et al., 1996; Xu et al., 1997; Fortun et al., 2009; Bunge and Wood, 2012). Despite the limited axonal growth associated with those treatments, axons regenerating through SC-seeded guidance channels have been shown to produce measurable evoked potentials in response to electrical stimulation in a small proportion of treated rats, and it is interesting to note that the animals that displayed evoked potentials were the ones that had the most myelinated axons present in the graft (Pinzon et al., 2001).

1.6.9.4 Intraparenchymal transplantation of N-SCs following SCI

In contrast to the early work that focused mainly on characterizing the ability of N-SCs to promote regeneration following spinal cord transection or their capacity to remyelinate demyelinated/regenerating CNS axons, more recent work has focused to a larger extent on
establishing the efficacy of those cells as a treatment for SCI. As such, recent investigations have typically employed more clinically relevant approaches, including contusion injuries and delayed transplantation, and those studies have frequently included behavioural assessments (Tetzlaff et al., 2011).

In light of their limited migration following transplantation into intact CNS parenchyma, cultured N-SCs are typically delivered directly into the lesion cavity when transplanted into the injured CNS parenchyma. Although N-SCs can survive following transplantation into the lesion cavity after SCI, as one might expect, their survival is compromised in that hostile environment (Hill et al., 2006; Hill et al., 2007). The survival of transplanted N-SCs is significantly improved if transplantation is delayed until 7 days post-injury, and for cell transplants into hosts that are not completely syngeneic, the addition of immunosuppression (e.g., CsA) further improves survival following delayed transplantation (Hill et al., 2006). However, even with delayed transplantation, the majority of N-SCs die after grafting into thoracic contusion injuries, where approximately ~80% of the transplanted cells are lost within the first week (Hill et al., 2007). The majority of cell death occurs within 24 hours of transplantation and although some cells undergo apoptosis, six times as many N-SCs die by necrosis (Hill et al., 2007).

In the absence of additional treatments, and despite their relatively poor survival, N-SCs transplanted into the injured spinal cord have consistently been found to partially fill/bridge the lesion cavity, increase white matter sparing, enhance the endogenous SC response, remyelinate axons of CNS origin and promote the growth/sprouting of CNS axons using a variety of animal models of SCI (Martin et al., 1991; Paino and Bunge, 1991; Martin et al., 1993; Li and Raisman, 1994; Paino et al., 1994; Martin et al., 1996; Imaizumi et al., 2000; Takami et al., 2002; Barakat
et al., 2005; Hill et al., 2006; Pearse et al., 2007; Schaal et al., 2007; Sharp et al., 2012). Much like the findings for N-SCs transplanted into demyelinated CNS lesions, those cells generally fail to migrate out of the lesion site following delayed transplantation and display limited integration with astrocyte-rich host tissue (Barakat et al., 2005; Andrews and Stelzner, 2007; Pearse et al., 2007; Schaal et al., 2007). However, transplanted N-SCs are able to bridge even relatively large lesion cavities, such as those produced by a moderate contusion, and within those bridges they form channel- or tube-like structures resembling the bands of Büngner found in denervated peripheral nerve (see examples in: Andrews and Stelzner, 2007; Pearse et al., 2007); thus providing an oriented substrate for the support of axonal growth into, and potentially through, the lesion site.

It is not uncommon for those bridges to contain tens of thousands of axons at their midpoint, even in the absence of co-treatments, whereas that region is usually occupied by a large cavity entirely devoid of axons in the absence of transplanted cells (see SC-only control values in: Pearse et al., 2004b; Golden et al., 2007). As in the transection studies, the majority of those axons are assumed to be propriospinal or sensory, as only a small number of those fibres are typically 5-hydroxytryptamine (5-HT)- or dopamine β-hydroxylase (DβH)-positive (suggesting brainstem raphespinal or coerulospinal origins) and axon tracing typically reveals very limited, if any, penetration of SC grafts by descending corticospinal, reticulospinal, or vestibulospinal axons (Martin et al., 1996; Takami et al., 2002; Pearse et al., 2004b; Barakat et al., 2005; Golden et al., 2007; Pearse et al., 2007; Schaal et al., 2007). The lack of robust supraspinal axon growth and the inability to promote regeneration across the distal graft-host interface have largely been confirmed using N-SCs alone in other SCI models as well, including
dorsal column transections (Imaizumi et al., 2000), photochemical lesions (Paino and Bunge, 1991; Paino et al., 1994), and subdural balloon compression (Martin et al., 1991; Martin et al., 1993). Despite those limitations, and in the absence of co-treatments, transplanted N-SCs have been found to restore rapid and secure conduction across dorsal column transections (Imaizumi et al., 2000), facilitate moderate recovery of locomotor function in multiple models of SCI (Takami et al., 2002; Schaal et al., 2007; Sharp et al., 2012), and even demonstrate some functional efficacy in the chronic injury setting (Barakat et al., 2005).

1.6.9.5 Summary of the benefits and limitations associated with N-SC transplantation following SCI

Although N-SC transplantation following SCI is associated with neuroprotection, tissue preservation, remyelination, and a modicum of axonal regeneration, on their own those cells are not able to completely reverse the damage caused by SCI and they generally provide only modest improvements in locomotor function (reviewed in: Fouad et al., 2005; Fortun et al., 2009; Tetzlaff et al., 2011; Bunge and Wood, 2012). The efficacy of N-SCs as a treatment for SCI appears to be restricted by a number of limitations, including: limited cell survival following transplanted into the lesion site; limited migration through and integration with astrocyte-rich CNS parenchyma; limited support of supraspinal axon growth; and limited regeneration across the distal graft-host interface (reviewed in: Oudega and Xu, 2006; Fortun et al., 2009; Bunge and Wood, 2012; Williams and Bunge, 2012). However, it is important to point out that those limitations are by no means specific to N-SCs alone. For example, the survival of cells transplanted into the injured CNS is a common concern for most exogenous cellular therapies (Hofstetter et al., 2002; Fan et al., 2008; Hill et al., 2010), and even cells that are known to elicit
little reactive astrogliosis and intermingle freely with astrocytes (e.g., OECs) tend to show very limited migration through intact CNS tissue (Lakatos et al., 2003; Pearse et al., 2007). Limited axonal growth and functional recovery are also common attributes of most cellular therapies, particularly in the absence of co-treatments (Oudega and Xu, 2006; Tetzlaff et al., 2011; Blesch et al., 2012). Furthermore, limited therapeutic efficacy is by no means specific to cellular therapies, as all of the non-cellular pharmaceutical and biological interventions tested to date have also provided only limited neuroprotection and/or neurorepair, and no single therapy has yet been discovered that completely reverses the loss of neurological functions that generally accompanies SCI (Bunge, 2001; Ramer et al., 2005; Bunge, 2008; Kuffler, 2012; Oudega et al., 2012). In light of all of that, combinatorial approaches have become the norm in SCI treatment research, as it is generally believed that combining multiple complimentary treatments is likely to provide maximal therapeutic benefit.

1.6.10 Overcoming the limitations of N-SCs to enhance CNS repair

Towards the goal of finding effective combinatorial treatments for SCI, much of the preclinical SCI research involving N-SCs has focused on finding co-treatments to overcome the limitations imposed on N-SCs transplanted into the injured CNS in order to maximize the therapeutic efficacy associated with those cells (reviewed in: Oudega and Xu, 2006; Fortun et al., 2009; Bunge and Wood, 2012; Williams and Bunge, 2012). Most of that effort has been directed at promoting the growth of supraspinal axons and axonal growth in general across the distal graft-host interface in the presence of grafts containing N-SCs via co-treatments designed to either promote, or reduce the inhibition of, axonal growth (see below). However, there has also been significant interest in improving the migration and integration of N-SCs into astrocyte-rich
CNS parenchyma, and for that purpose, *in vitro* models such as the inverted coverslip migration assay and the boundary assay (Afshari et al., 2011) were developed to facilitate the study of the mechanisms underlying the interactions of N-SCs and astrocytes. In contrast to those two areas of interest, relatively little work has been conducted to directly address the limited survival of N-SCs transplanted into the CNS.

### 1.6.10.1 Improving the survival of N-SCs in the injured spinal cord

Given that only ~20% of N-SCs survive beyond the first week *in vivo* following delayed transplantation (1 week to 2 months post-injury) into the contused thoracic spinal cord of the rat (Barakat et al., 2005; Hill et al., 2007; Pearse et al., 2007), it would appear that there is plenty of room to improve the survival of N-SCs transplanted into the injured CNS. However, the fact that N-SCs are known to increase the presence of endogenous SCs in the injured spinal cord raises the question of whether or not improving the survival of transplanted N-SCs above 20% will actually improve the outcome of N-SC transplantation. Following N-SC transplantation into thoracic contusions, endogenous SCs appear to replace the transplanted cells that are lost due to cell death, and those endogenous cells generally outnumber the transplanted cells by a substantial margin (2-3X) at long-term endpoints even after delayed transplantation and in the presence of immunosuppression (Hill et al., 2006; Hill et al., 2007; Pearse et al., 2007). Thus it may be the case that improving the survival of transplanted cells will merely reduce the substrate (e.g., denuded axons) available for endogenous SCs and the total number of SCs in the cord may remain unchanged. Regardless of whether or not that is the case there are other reasons why it may be desirable to prevent the death of transplanted N-SCs. For example, preventing the necrotic death of N-SCs should limit any additional tissue damage caused by the rupture and
death of those cells and would not only reduce the number of cells required for transplantation, but would also reduce the loss of genetically modified cells when N-SCs are used as a vector for gene therapy (Hill et al., 2007). To date the search for effective co-treatments to improve N-SC survival in vivo has received relatively little attention, but towards that goal, recent work has demonstrated that the inclusion of a calpain inhibitor in the suspension media doubled the number of those cells present 1 week after transplantation into the contused thoracic spinal cord of the rat (Hill et al., 2010). In addition, the genetic modification of N-SCs to overexpress neurotrophins (e.g., D15A [mimics actions of NT-3 and BDNF] or NGF) and systemic administration of Rolipram (to elevate intracellular cAMP) have also been associated with increased survival of N-SCs following transplantation into the injured spinal cord (Weidner et al., 1999; Golden et al., 2007; Flora et al., 2012).

1.6.10.2 Improving N-SC interactions with astrocytes

The limited migration/integration of N-SCs into astrocyte-rich host tissue is a primary issue of concern for the potential application of those cells as a therapy for MS, but it may also have important implications for the use of those cells as a therapy for SCI, as the inability of N-SCs to migrate out of the lesion site prevents those cells from myelinating denuded axons in the adjacent, relatively intact host tissue and keeps them from interacting with injured axons that have retracted from the lesion site. In addition, the lack of proper integration with host astrocytes at the lesion-graft border is thought to a play role in limiting the ability of N-SCs to promote axonal growth across the distal graft-host interface (Lakatos et al., 2003; Afshari et al., 2010a). As such, there is a great deal of interest in improving the interactions between N-SCs and astrocytes with the goal of enhancing the therapeutic efficacy of N-SCs in SCI.
The transplantation of N-SCs or PN into intact CNS tissue elicits astrocyte hypertrophy and increased GFAP expression and the resulting astrocytic glial scar is thought to play a significant role in impeding the migration of SCs out of the site of implantation and thereby preventing their integration into the adjacent CNS parenchyma (Fishman et al., 1983; Blakemore et al., 1986; Sims et al., 1999; Lakatos et al., 2003; Andrews and Stelzner, 2007). Research examining the interactions of N-SCs and astrocytes in co-culture assays has demonstrated similar effects, as those cells spontaneously segregate into non-overlapping compartments with sharp boundaries between them \textit{in vitro} and N-SCs display very limited migration over astrocyte monolayers (Wilby et al., 1999; Lakatos et al., 2000; Fairless et al., 2005; Grimpe et al., 2005; Santos-Silva et al., 2007; Afshari et al., 2010a; Afshari et al., 2010b). Furthermore, exposure to N-SCs was found to induce hypertrophy and increased CSPG expression in astrocytes (Lakatos et al., 2000; Grimpe et al., 2005; Santos-Silva et al., 2007).

To date those \textit{in vitro} models have found evidence that a variety of molecules mediate the undesirable interactions between N-SCs and astrocytes and a number of interventions have been shown to promote favourable interactions between those two cell types. For example, blocking NCad or Eph receptors on N-SCs (Wilby et al., 1999; Fairless et al., 2005; Afshari et al., 2010a), activating N-SC integrins or blocking astrocyte-production of aggrecan all enhance N-SC migration over astrocyte monolayers (Afshari et al., 2010b), whereas degrading CSPGs or heparin sulfate proteoglycans (HSPGs) (Grimpe et al., 2005; Santos-Silva et al., 2007), blocking Ephrin signalling (Afshari et al., 2010a), adding exogenous GDNF (Deng et al., 2011) or genetically modifying N-SCs to express polysialylated NCAM (PSA-NCAM) (Papastefanaki et al., 2007; Luo et al., 2011) all increase the intermingling between N-SCs and astrocytes, and
suppressing FGF production by N-SCs (Santos-Silva et al., 2007) or adding exogenous GDNF (Deng et al., 2011) reduces the degree of astrocyte reactivity they induce.

Of those potential interventions, only the genetic modification of N-SCs to express PSA-NCAM has been linked to enhanced migration of N-SCs through astrocyte-rich host tissue in vivo, as (Luo et al., 2011) found that the presence of PSA-NCAM on N-SCs improved the migration of mouse N-SCs from intact tissue into a nearby crush injury, and (Ghosh et al., 2012) demonstrated that the majority of rat N-SCs expressing PSA-NCAM migrated out of thoracic contusion sites to intermingle with astrocytes in the spared tissue (up to 4.4mm away) and were replaced in the lesion site by endogenous SCs. The enhanced migration/integration of N-SCs expressing PSA-NCAM into spared host tissue appeared to have the desired effect, as treatment with those cells was associated with enhanced serotonergic and corticospinal axon regeneration across the caudal graft-host interface as well as significant behavioural recovery in rats with thoracic contusion (Ghosh et al., 2012). Similar findings have been reported for N-SCs engineered to express PSA-NCAM transplanted into the focally demyelinated CNS, as those cells migrated more rapidly to remyelinate a remote lesion site (Bachelin et al., 2010). The degradation of CSPGs with chondroitinase ABC (ChatABC; see below) and the application of exogenous GDNF have also been tested in vivo, and although both of those treatments reduced the presence of inhibitory CSPGs at the graft-host interface, neither was found to enhance the migration of N-SCs out of mini-channels transplanted into the hemisected thoracic spinal cord of the rat (Chau et al., 2004; Deng et al., 2011).
1.6.10.3 Co-treatments to enhance the growth of CNS axons in general

A wide variety of combinatorial strategies have been tested in efforts to improve the regeneration associated with N-SC transplantation, but probably the most popular approach in preclinical research has been to elevate neurotrophic factor levels in the injured spinal cord. Two main strategies have been utilized to accomplish that task: direct infusion of exogenous factors (see examples in: Xu et al., 1995b; Iannotti et al., 2003); and the genetic modification of N-SCs to induce overexpression of particular neurotrophins (see examples in: Tuszynski et al., 1998; Golden et al., 2007). Using those approaches, we have learned a great deal about which CNS axon populations respond to which neurotrophic factors, and although there is overlap in that regard, there is also a substantial amount of variability in terms of the amount of growth promoted.

The elevation of GDNF, NT3 and/or BDNF are all associated with the enhanced growth of propriospinal axons into N-SC grafts (Xu et al., 1995b; Menei et al., 1998; Bamber et al., 2001; Golden et al., 2007), whereas the elevation of BDNF or NGF generally improve sensory axon growth (Oudega and Hagg, 1996; Menei et al., 1998; Tuszynski et al., 1998; Weidner et al., 1999). The growth of various brainstem-spinal (e.g., reticulospinal, vestibulospinal, or raphespinal) axons into N-SC or PN grafts is enhanced by elevating GDNF, NT3 and/or BDNF (Xu et al., 1995b; Menei et al., 1998; Dolbeare and Houle, 2003), whereas coeruleospinal axons in particular are known to respond to N-SCs genetically modified to overexpress NGF (Tuszynski et al., 1998; Weidner et al., 1999). In terms of major descending motor tracts in rodents, exogenous GDNF is known to enhance the sprouting of RST axons into PN grafts (Dolbeare and
Houle, 2003), but the CST has proven to be largely resistant to the combination of grafts containing N-SCs and elevated neurotrophic factors alone (see below).

It should be noted that neurotrophic factors are not the only co-treatments that elicit enhanced growth from specific axon populations in combination with N-SCs. For example, the elevation of cAMP has been reported to promote the growth of serotonergic fibres as well as multiple brainstem-spinal axon populations (Pearse et al., 2004b; Flora et al., 2012), whereas the transplantation of OEG outside of N-SC grafts is linked with increased serotonergic and reticulospinal axon growth in particular (Ramon-Cueto et al., 1998; Pearse et al., 2004a). Although increasing axonal growth sounds beneficial, that growth may not be functionally relevant if the fibres fail to exit the graft and re-enter the spared host tissue distal to the injury site.

### 1.6.10.4 Promoting regeneration across the distal graft-host interface

Limited axon growth across the distal graft-host interface following N-SC transplantation is thought to be largely due to the formation of the glial scar and the accumulation of inhibitory proteoglycans (Plant et al., 2001; Hsu and Xu, 2005). Transplanted N-SCs are known to enhance astrocyte reactivity over and above that already elicited by the injury itself, particularly at the caudal graft-host interface (Plant et al., 2001), and thus it would seem that the response of host astrocytes to transplanted N-SCs contributes to the failure of axonal regeneration across the distal graft-host interface (Lakatos et al., 2003). Support for this notion comes from the fact that other cell types (e.g., SCPs and OECs) that are known to induce less reactive astrogliosis and CSPG expression by astrocytes are also known to support enhanced axonal regeneration across the graft-host interface following transplantation into the injured CNS (Lakatos et al., 2000;
Evidence that CSPGs play an important role in limiting the growth of CNS axons out of grafts containing N-SCs comes from a number of studies that have demonstrated that the application of the enzyme ChatABC (which cleaves the sulfated glycosaminoglycan sidechains that inhibit axonal growth from CSPGs) at the graft-host interface facilitates the growth of propriospinal axons into the spared host tissue distal to the graft (Yick et al., 2000; Chau et al., 2004; Fouad et al., 2005; Vavrek et al., 2007; Tom et al., 2009; Kanno et al., 2012). Although that treatment does not appear to promote the migration of N-SCs, it does promote the migration of host astrocytes into N-SC-seeded channels, and those cells extend processes parallel to the axis of the bridge that support the growth of regenerating propriospinal axons across the graft-host interface (Chau et al., 2004). Curiously, similar effects were obtained when the levels of GDNF were elevated in conduits containing N-SCs, either by direct infusion of GDNF or genetic modification of the N-SCs to overexpress that factor (Deng et al., 2011).

Other methods that appear to be effective at enhancing the growth of CNS axons out of N-SC grafts include the direct infusion of neurotrophic factors (e.g., BDNF and/or NT3) (Bamber et al., 2001), the elevation of intracellular cAMP (Pearse et al., 2004b; Flora et al., 2012), or the transplantation of OEG in the parenchyma caudal to the injury site (Ramon-Cueto et al., 1998; Pearse et al., 2004a).

1.6.10.5 Maximizing the therapeutic efficacy of N-SC therapy for SCI

As one might expect, combinatorial strategies that include multiple co-treatments have proven most effective in promoting supraspinal axon growth, regeneration across the distal graft-
host interface, and functional recovery following SCI. The earliest example of such a strategy comes from the PN graft literature as one might expect. In that work fibrin glue containing FGF-1 was used to stabilize multiple PN grafts connecting the rostral and caudal stumps of the fully transected spinal cord in conjunction with vertebral stabilization (Cheng et al., 1996). Retrograde tracing from the lumbar enlargement 1 year later indicated that successful regeneration into the gray matter caudal to the injury site had been achieved for numerous brainstem-spinal (reticulospinal, rubrospinal and raphespinal) axon populations, as well as the CST, and that regeneration resulted in significant improvements in hindlimb locomotor function (Cheng et al., 1996). That work has since been replicated in the same model, where it was demonstrated using multiple linear regression that the recovery of locomotor function only correlated with the regeneration of the CST into the lumbar gray matter (Tsai et al., 2005). That procedure has also been shown to improve motor functions in a single human patient with chronic paraplegia (Cheng et al., 2004). Using a similar approach, Guest et al. (1997b) applied fibrin glue containing FGF-1 to stabilize channels containing cultured N-SCs between the stumps of the fully transected spinal cord and also managed to elicit the ingrowth of the CST, but unlike propriospinal and sensory fibres that also grew in, the CST fibres failed to exit the graft and re-enter caudal host tissue.

Another approach that has shown some promise is the combination of an N-SC graft, systemic elevation of intracellular cAMP using Rolipram, and direct injections of dibuteryl cAMP near the graft; which was shown to prompt serotonergic axons to exit the graft at the caudal interface and resulted in a significant improvement in hindlimb locomotor recovery (Pearse et al., 2004b). In a more recent study, Rolipram was combined with the transplantation of
N-SCs genetically modified to express D15A (a bi-functional neurotrophic factor that mimics the actions of BDNF and NT3) into thoracic contusion sites and the full combination of D15A-expressing N-SCs and Rolipram provided the highest number of serotonergic axons in the graft, the most reticulospinal axons in the caudal spinal cord and significantly improved base of support as well as paw rotation on the CatWalk device compared to Rolipram combined with normal N-SCs (Flora et al., 2012).

ChatABC has become a very popular treatment to include in combinatorial approaches with N-SCs. For example, in one study, the combination of OEG and ChatABC applied to either end of a N-SC-seeded bridge applied to the fully transected thoracic spinal cord has been shown to enhance the regeneration of a variety of brainstem axon populations (reticular, vestibular, and raphe nuclei) as well as propriospinal axons through the bridge and into the lumbar spinal cord (Vavrek et al., 2007), and in a second study using the same approach the number of myelinated axons in the bridge was positively correlated with improved locomotor functions (Fouad et al., 2005). Similarly, the application of exogenous GDNF and ChatABC was found to facilitate the growth of brainstem-spinal axons growth beyond a PN graft and also improved functional recovery (Tom et al., 2009). And finally, when transplanted into contusion sites in the rat, N-SCs modified to secrete chondroitinase and the bi-functional D15A neurotrophic factor were shown to increase the ingrowth of brainstem-spinal axons, enhance CST sparing, and promote the exit of propriospinal axons from the graft (Kanno et al., 2012; Williams and Bunge, 2012). Furthermore, those cells improved locomotor and sensory recovery, and the combination of both chondroitinase and D15A expression was superior to either of those factors alone.
1.6.11 Clinical trials of N-SC transplantation following SCI

Despite the ongoing preclinical efforts to establish effective co-treatments to maximize the neurological recovery associated with N-SC transplantation, autologous N-SCs are already being examined as a therapy for SCI in human clinical trials. Clinical transplantation of N-SCs has been ongoing for some time in China, with unpublished (Tator, 2006) and published (Xian-Hu et al., 2012) anecdotal reports indicating neurological improvement in some patients, and two reports from an ongoing clinical trial of N-SC transplantation have now been published by clinicians in Iran (Saberi et al., 2008; Saberi et al., 2011). In the latter work, autologous N-SCs harvested from the sural nerve of patients were transplanted directly into the spinal cords of patients with chronic (>2 years post-injury), non-penetrating, complete (ASIA A) or severe incomplete (ASIA B), thoracic or cervical SCI. By the time of the second report, 33 patients were enrolled in the study and had been tested for at least 2 years post-treatment (i.e., at least 4 years post-injury).

The findings from that work indicate that SC transplantation is safe, well-tolerated, and associated with significant yet modest neurological improvements, including improved light touch sensation and motor function, although the latter only reached significance in patients with cervical injuries that occurred <3 years prior to treatment. A small number of patients also reported improvements in autonomic bowel and bladder functions, and again those improvements were more prominent in patients with cervical injuries. To attain any improvements in patients with chronic SCI is rare, so these results are considered extremely promising, although additional large-scale testing and placebo controls will be required to definitively determine the efficacy of autologous N-SC transplantation. It is important to point
out that this treatment should probably be considered a combinatorial therapy, as extensive and continuous rehabilitation was conducted throughout the study. But it is equally important to note that all patients were at least 2 years post-injury and had undergone at least 6 months of rehabilitation to ensure spontaneous recovery had reached a plateau prior to study enrolment. In light of the preliminary clinical reports of feasibility, safety, and some benefit, as well as evidence of efficacy from decades of preclinical animal work, the Miami Project to Cure Paralysis has now initiated its own clinical trial of N-SC therapy in the US (http://miamiproject.miami.edu), which was recently granted FDA approval (Talan, 2012).

1.6.12 Drawbacks of autologous N-SC application

A significant drawback to the autologous clinical application of N-SCs or peripheral nerve grafts is that the generation of those cells/grafts can only be accomplished via the invasive surgical excision of segments of peripheral nerve. That procedure invariably causes peripheral nerve injury, which is associated with deficits or abnormalities in PNS neurological function, the nature, severity and persistence of which vary depending on the particular nerve(s) selected and the size of the nerve segment that is harvested. The size of the nerve segment harvested is relevant because the generation of nerve grafts/cells often requires the removal of a substantial length of whole nerve; 17-44cm for nerve grafts depending on the nerve being repaired and the size of the defect (Martins et al., 2012) or 12-15cm to generate N-SCs for transplantation (Saberi et al., 2011). As such, the excision of nerve for those purposes typically creates an injury with a large discontinuous nerve gap completely devoid of SC basal lamina; which is precisely the kind of injury that practically guarantees the failure of spontaneous PNS regeneration. Indeed, even if the nerve stumps are reconnected using a simple conduit, spontaneous regeneration typically
fails for gaps >1cm in rat and >3cm in primates without the addition of exogenous SCs or other growth promoting extrinsic factors (reviewed in Hood et al., 2009), and if a length of about 10cm of nerve is removed in humans, even immediate repair with an autologous nerve graft would be expected to fail (Kline, 2004).

In order to minimize the resulting functional deficits, clinicians typically select ‘non-critical’ sensory nerves for harvest, and the sural nerve has been the most popular choice for peripheral nerve reconstruction in humans for decades, due to its easily accessible anatomic location, relatively thick diameter, and length (up to 43cm on average available for harvest in adult humans) (Yavuzer et al., 2002; Wolford and Stevao, 2003; Riedl et al., 2008; Hood et al., 2009). Not surprisingly, the sural nerve was also selected by Saberi and colleagues (2008; 2011) to generate N-SCs for their SCI clinical trials, and a similar approach is planned for the upcoming trials being conducted by the Miami Project to Cure Paralysis (http://miamiproject.miami.edu).

In humans, the sural nerve is one of the major sensory nerves providing cutaneous sensation to the lower limb; particularly the posterior and lateral aspects of the distal third of the leg, including the lateral heel, the lateral aspect of the foot and the small toe (Aktan Ikiz et al., 2005). Except for some unmyelinated autonomic fibres, the nerve is entirely sensory (Lawrence and Botte, 1994), so its excision carries no direct consequences to motor function per se and typically results in sensory morbidity that is limited to a ‘relatively unimportant’ (i.e., non-weight-bearing) part of the foot (Kline, 2004). Although sural nerve excision typically only results in diminished sensitivity to stimuli (i.e., hypoesthesia) or a complete loss of all sensory modalities (i.e., anesthesia) in the region normally innervated by the nerve, some patients
experience other forms of sensory morbidity, including spontaneous or evoked abnormal sensations (i.e., paresthesia) such as tickling, tingling, burning, pricking or numbness, commonly referred to as the feeling of “pins and needles” or a limb “falling asleep”, which can be unpleasant or even painful (i.e., dysesthesia), and in rare cases those symptoms can develop into neuropathic pain that occurs either in response to stimulation (e.g., allodynia or hyperalgesia) or spontaneously (Pollock et al., 1983; Theriault et al., 1998; Huang and Zager, 2004; Schoeller et al., 2004).

Immediately after the harvest of health sural nerve for grafting, virtually all patients report a noticeable loss of sensation in the region of sural innervation, but over time the degree of hypoesthesia and/or the size of the affected area often decreases (Miloro and Stoner, 2005; IJpma et al., 2006; Martins et al., 2012) due to the combined effects of collateral sprouting in adjacent cutaneous nerves and reorganization of the cortical representation of the sensory area (Aszmann et al., 1996; Cusick, 1996; Theriault et al., 1998). Despite those improvements, a noticeable sensory deficit generally persists for all patients in the first 1-3 years post-surgery (Miloro and Stoner, 2005; Martins et al., 2012), and in the majority of patients (76%) at follow-ups ranging from 16-34 years post-surgery (IJpma et al., 2006). Thus, for the majority of patients, sural nerve harvest for grafting results in a noticeable sensory deficit that appears to be permanent, and although the resulting sensory deficit is generally thought to be of little functional significance (Kline, 2004), it is of interest to note that there is some evidence to indicate that sural nerve harvest for grafting can result in an altered gait pattern (Yavuzer et al., 2002).

Unlike the consistency found in assessments of sensory losses following sural nerve harvest for grafting, the occurrence of paresthesia, dysesthesia, and pain vary widely from study-
to-study. Only five studies have directly assessed donor site morbidity following the harvest of healthy sural nerve for peripheral nerve reconstruction (Staniforth and Fisher, 1978; Miloro and Stoner, 2005; IJpma et al., 2006; Lapid et al., 2007; Martins et al., 2012), and in those studies, the age of participants, length of sural nerve removed, time since surgery, surgical techniques, and measures of morbidity all varied substantially, making it virtually impossible to accurately estimate the incidence or prevalence of paresthesia, dysesthesia, and pain in that population from the available literature. What can be drawn from those studies however, is that sensory morbidity is typically mild, and may occur in many patients early after sural nerve excision, but tends to spontaneously resolve within months or years post-surgery (Miloro and Stoner, 2005; IJpma et al., 2006; Lapid et al., 2007; Martins et al., 2012). There are of course some exceptions, as mild symptoms of numbness, cold sensitivity, and pain have been reported at follow-ups ranging from 3-34 years in a small number of patients, but for the vast majority of patients, the sensory morbidity associated with sural nerve harvest is minimal, and does not have a significant effect on their ability to perform daily activities (Miloro and Stoner, 2005; IJpma et al., 2006; Lapid et al., 2007; Martins et al., 2012). Similar findings have been reported when sensory morbidity is assessed following the biopsy of sural nerve to diagnose peripheral neuropathy; which is a much more common procedure that has been more extensively studied (Thomas, 1970; Pollock et al., 1983; Poburski et al., 1985; Solders, 1988; Neundorfer et al., 1990; Perry and Bril, 1994; Flachenecker et al., 1999; Gabriel et al., 2000; Kline, 2004; Ruth et al., 2005). However, in contrast to that literature, there are reports that indicate that some patients experience persistent dysesthesia and/or neuropathic pain that is severe enough to warrant medical attention (and even surgical intervention) following the resection of sural nerve for the purposes of autologous PNS reconstruction (Brunelli, 2002; Wolford and Stevao, 2003) or histopathological testing (Poburski
et al., 1985; Solders, 1988). Thus for a very small number of patients, the resection of sural nerve has serious and detrimental consequences.

The occurrence of paresthesia, dysesthesia and neuropathic pain following nerve transection are thought to result primarily from aberrant signals related to abortive regeneration and neuroma formation (Lindenlaub and Sommer, 2000; Zeltser et al., 2000; Zimmermann, 2001). Severed peripheral axons automatically attempt to regenerate, but when regeneration fails, nerve fibres tend to grow out in all directions from the proximal stump due to a lack of appropriate guidance (Zimmermann, 2001; Edstrom and Karacaoglu, 2004). Meanwhile, fibroblasts in the proximal nerve stump proliferate and deposit collagen and other ECM molecules that harden over time to form a connective tissue scar, and the combination of intraneural fibrosis and disorganized growth of nerve fibres results in the formation of a bulb-like mass or nodule at the proximal nerve stump that is known as a neuroma (Lindenlaub and Sommer, 2000; Zimmermann, 2001; Brunelli, 2002; Edstrom and Karacaoglu, 2004). Neuroma formation is an inevitable consequence of peripheral nerve transection in the absence of repair or successful regeneration (Zeltser et al., 2000; Koch et al., 2003; Edstrom and Karacaoglu, 2004), and although the majority of neuromas are relatively asymptomatic, it has been estimated that 20-30% of all neuromas become painful (Herndon, 1982; Nath and Mackinnon, 1996). Painful neuromas are often exquisitely sensitive to touch or percussion and that mechanical sensitivity can persist for months or years after PNS injury, and in rare cases, that pain can evolve into spontaneous persistent neuropathic pain (Zeltser et al., 2000; Zimmermann, 2001; Edstrom and Karacaoglu, 2004). Thus, although the risk of developing a painful neuroma is rather low following nerve resection, the potential consequences of that occurrence can be quite severe.
In light of the potential development of neuropathic pain, much effort has been directed towards the development of surgical techniques to prevent the formation of painful neuromas following the surgical transection of peripheral nerves. Indeed, over 150 techniques for resecting nerve were tested in humans and animals prior to 2000 (Zeltser et al., 2000), and although some success has been demonstrated for many of those techniques alone or in combination, no procedure has thus far proven to completely and consistently prevent neuroma formation at the proximal stump of a transected nerve (Edstrom and Karacaoglu, 2004; Huang and Zager, 2004); save for the destruction of the nerve cell body, which completely inhibits regeneration and thus neuroma formation (Guttmann and Medawar, 1942).

Given the lack of successful treatments or prevention measures, the risk of neuroma formation is often cited as one of the primary reasons to avoid surgical nerve resection unless absolutely necessary. With respect to PNS repair, the desire to avoid those risks, has helped to drive a substantial amount of research aimed at developing alternative conduits (e.g., autologous or cadaveric vein, artery, muscle or bone conduits; laminin, fibronectin and collagen conduits; and synthetic conduits made of various polymers) to replace autologous peripheral nerve grafts (reviewed in: Evans, 2000; Meek and Coert, 2002; Wolford and Stevao, 2003; Hood et al., 2009). Meanwhile, despite over 40 years of widespread use as a diagnostic tool, many clinicians today call for caution in the use of sural nerve biopsy due in part to the risk of serious sensory morbidity, such as neuropathic pain (Suarez et al., 2001; Ruth et al., 2005). Indeed, it has been suggested that nerve biopsy should be treated as a last resort for diagnosis, and used only in situations where the etiology of the underlying neuropathy remains unclear after all of the available, less invasive neurological tests (e.g., CT scans, MRI, electromyography (EMG), nerve
conduction velocity tests) have been exhausted (Gabriel et al., 2000; Ruth et al., 2005). Of significant relevance to the present work, is the fact that skin biopsy is one of the diagnostic tests for peripheral neuropathy that is suggested prior to resorting to nerve biopsy in many cases, as it is less invasive, less painful, and has fewer negative side effects in comparison to nerve biopsy (Lauria and Devigili, 2007).

With respect to the use of autologous N-SCs or peripheral nerve grafts in therapeutic clinical applications, it is generally assumed that the benefits of those treatments will outweigh the loss of peripheral sensation and the risk of serious sensory morbidity. This is particularly true for people with SCI, as many of those individuals already have substantial sensory deficits, abnormal sensations and pain as a result of central damage, so they may be unlikely to even notice the effects of an injury to the sural nerve. However, given that the primary goal of treating SCI is to improve the recovery of lost functions, and that N-SC treatment may provide some recovery of peripheral sensation, it seems unwise to assume that patients treated with those cells will not recover enough function to experience sensory morbidity resulting from sural nerve excision. Furthermore, not all humans with SCI have complete loss of sensation in the region innervated by the sural nerve to begin with, so a proportion of the patient population should be expected to be susceptible to those negative effects regardless of the outcome of treatment.

The harvest of peripheral nerve is also a relatively invasive surgery, and although surgical complications (e.g., infections, hematoma or deep vein thrombosis) are quite rare when nerve is harvested from healthy donors (Staniforth and Fisher, 1978; Miloro and Stoner, 2005; Ijpma et al., 2006; Martins et al., 2012), the risk of such complications is likely to be higher in the SCI population given that SCI is known to negatively impact circulatory and immune functions.
(Schwab et al., 2006; Casha and Christie, 2011). Similarly, neuropathic pain is already quite common in SCI (Gustin et al., 2010), so although the risk of painful neuroma formation after sural nerve harvest is low, if aberrant pain signalling does result from that procedure it may hasten or worsen the development of central pain (Zimmermann, 2001). Furthermore, the fact that sural nerve excision has been shown to alter gait in otherwise healthy individuals (Yavuzer et al., 2002) raises the possibility that the loss of sensory function in that region may complicate the recovery of locomotor functions for patients with SCI.

In summary, our current knowledge of the consequences and risks associated with sural nerve harvest in individuals with SCI is extremely limited, and although that procedure appears to be safe and cause little discomfort for most people without SCI, there remains a risk of serious sensory morbidity and other complications that may limit or negatively influence the recovery of sensory/motor function following N-SC transplantation to treat SCI. In an effort to avoid those risks, there has been considerable interest in finding a source of autologous SCs other than peripheral nerve, and a variety of stem/precursor cells that can be isolated from non-neural tissue have been suggested as potential alternatives to autologous N-SCs for therapeutic clinical application to treat CNS injury/disease.

### 1.7 Alternative sources of autologous SCs

Without resorting to ES or iPS cells, or attempting to directly reprogram somatic cells, there are four main sources of autologous SCs other than peripheral nerve that have been identified in adult mammalian tissues to date. This thesis focuses on one of those sources, SKP-SCs generated from precursors (SKPs) found in the dermis of adult mammalian skin. Two more
potential sources of autologous SCs are also found in adult mammalian skin: epidermal neural crest stem cells (EPI-NCSCs) (Sieber-Blum et al., 2004) and hair follicle pluripotent stem (hfPS) cells (Amoh et al., 2005). And the final source of cells are MSCs found in a variety tissues, which have been reported to transdifferentiate into an SC-like state under appropriate conditions in culture (Kuroda et al., 2011).

To my knowledge EPI-NCSCs and hfPS cells have yet to be differentiated, purified and expanded in vitro to be used as a source of SCs for therapeutic applications, as those cells are generally transplanted as naïve stem cells. Although follicular stem cells are reported to ‘mostly’ generate GFAP- and CNPase-positive SCs following transplantation into the injured spinal cord (Amoh et al., 2008), CNS glia also express those markers, so there is little solid evidence that those cells differentiate into SCs in vivo, and attempts to confirm that those cells produce SC myelin in the injured CNS are lacking to date. Meanwhile, EPI-NCSCs have been reported to differentiate into neurons and oligodendrocytes rather than SCs under similar conditions (Sieber-Blum et al., 2006; Hu et al., 2010). In light of all that, neither of those cell types seems poised to play a role as a replacement for N-SCs per se; though both may be considered alternative cellular therapies for SCI. In contrast, transdifferentiated MSCs (tMSCs) generated in vitro have received considerable preclinical attention with the express purpose of determining whether or not those cells represent a suitable alternative to autologous N-SCs for clinical SC-therapies. As such, it is worthwhile to address the question of whether those cells are in fact a suitable source of autologous SCs before we review the literature on SKPs and SKP-SCs.
1.7.1 Do tMSCs represent an alternative source of SCs for therapeutic applications?

A variety of mesenchymal stem cells (MSCs) isolated from bone marrow, umbilical cord, and adipose tissue have all been reported to ‘transdifferentiate’ to an SC-like state under specific culture conditions (Kuroda et al., 2011), and those cells have also been suggested as a potential replacement for autologous N-SCs in the clinic. *In vitro* tMSCs have been shown to take on an SC-like morphology, label with a variety of typical SC markers (e.g., p75, S100β, and GFAP) and express a myelinating SC phenotype (including expression of P0, PMP-22 and MBP) in co-culture with neurons (Caddick et al., 2006; Radtke et al., 2009; Mantovani et al., 2010; Peng et al., 2011). Those cells also produce a repertoire of growth factors similar to that displayed by N-SCs and have been shown to promote neurite outgrowth *in vitro* and moderate axonal growth/sprouting *in vivo* both in the injured PNS and CNS (Someya et al., 2008; Park et al., 2010; Ladak et al., 2011; Peng et al., 2011; Tomita et al., 2013). However, tMSCs appear to lack phenotypic stability, as their expression of SC markers is transient *in vitro* and their SC-like phenotype is reversible and generally requires continuous exposure to the transdifferentiation cytokine cocktail (typically containing basic FGF [bFGF], PDGF-AA, NRG1-β, IGF-1, and/or forskolin) in order to be maintained in culture (Keilhoff et al., 2006a; Keilhoff et al., 2006b; Ladak et al., 2011). One exception to that critique are the SC-like cells generated from adipose-derived stem cells (ASCs) that are grown as spheres (Radtke et al., 2009); but those cells have yet to be examined *in vivo*.

Although it has been proposed that exposure to injured axons *in vivo* stabilizes the SC-like phenotype (Keilhoff et al., 2006b), the assessments of myelination conducted following transplantation of those cells to date largely fail to provide convincing evidence of that, as
demonstrations of substantial numbers of cultured tMSCs producing SC-like myelin following transplantation into the injured PNS or CNS is generally absent from the literature. Typically studies involving those cells have either failed to label transplanted cells to rule out myelination by endogenous SCs (Keilhoff et al., 2006a; Keilhoff et al., 2006b; di Summa et al., 2011; Zaminy et al., 2013) or shown only limited evidence of myelination by one or two labelled cells (Kamada et al., 2005; Shimizu et al., 2007; Someya et al., 2008; Novikova et al., 2011; Tomita et al., 2012; Tomita et al., 2013). The latter work has also generally failed to provide convincing evidence that those labelled cells are actually producing a myelin sheath around an axon, largely due to a lack recognizable SC/myelin morphology in the depicted images. In fact, to my knowledge, only one study has shown such evidence by using immuno-EM to demonstrate a single tMSC-derived cell producing SC-like compact myelin around a regenerating PNS axon in vivo (Dezawa et al., 2001). Thus, there appears to be limited evidence that a handful of transplanted tMSCs can myelinate in vivo, but there is no evidence that SC-like myelination is a property shared by that population of cells in general. Furthermore, when tMSC myelination does occur it tends to be abnormal, as the myelin generated in denervated muscle grafts treated with unlabelled tMSCs and applied to the injured sciatic nerve was shown to be thinner than that produced by grafts treated with N-SCs, and unlike the latter, the tMSC-treated grafts often contained cells that appeared to myelinate multiple axons (Keilhoff et al., 2006a; Keilhoff et al., 2006b); which is a property quite distinct from the 1:1 myelination that is a defining characteristic of the SC phenotype.

Demonstrating that a handful of cells in a population are able to produce SC-like myelin in vivo is not adequate evidence to support the notion that the population generally possesses the
ability to do so; particularly when those cells are harvested as adherent cells from a niche such as bone marrow, which is known to house a small population of endogenous SCs (Yamazaki et al., 2011). Clearly there is a need for further research to demonstrate that tMSCs represent a stable population of cells that are able to produce myelin sheaths that are equivalent to those generated by endogenous SCs or transplanted N-SCs. In the absence of such evidence it is impossible to conclude that those cells represent a population of bona fide SCs, and therefore they cannot be considered a suitable replacement for N-SCs based on current evidence. That is not to say that tMSCs have no potential therapeutic benefit, as they generally appear to promote a modicum of axonal growth/sprouting and modest functional recovery in the injured spinal cord (Kamada et al., 2005; Someya et al., 2008; Novikova et al., 2011; Zaminy et al., 2013), so those cells remain a potential transplant candidate cell type, regardless of whether or not they represent a source of autologous SCs for clinical applications. The next obvious question is whether or not SKP-SCs represent a population of bona fide SCs, but before addressing that question, I will review what we know currently about the precursors from which those cells are generated.

1.8 SKPs as an alternative source of SCs for therapeutic applications

1.8.1 The isolation and characterization of SKPs

SKPs were initially isolated from the dermis of rodent and human skin by the Miller laboratory (University of Toronto) (Toma et al., 2001; Toma et al., 2005). The isolation of those cells is accomplished using a protocol that is akin to the neurosphere assay commonly used to isolate neural stem/progenitor cells from CNS tissue, but applied to skin, and much like NSPCs, non-adherent SKPs proliferate and self-renew as floating spheres in response to fibroblast growth
factor-2 (FGF-2) and epidermal growth factor (EGF) (Biernaskie et al., 2006; Hunt et al., 2009). Subsequent characterization of those cells revealed that they were multipotent and exhibited gene expression and differentiation properties similar to that of neural crest stem cells, including the ability to generate mesodermal derivatives such as adipocytes and skeletogenic cells and neural crest derivatives such as SCs and cells that resemble (morphologically and antigenically, if not functionally) peripheral neurons under appropriate conditions in vitro or in vivo (Toma et al., 2001; Fernandes et al., 2004; Toma et al., 2005; Fernandes et al., 2006; McKenzie et al., 2006; Lavoie et al., 2009). Furthermore, when grown as spheres in the presence of EGF and FGF-2, SKPs were found to retain their ability to proliferate and self-renew over multiple passages and even after a year in culture those cells retained multipotency (Toma et al., 2001). Based on those results it was suggested the SKPs represent a population of endogenous multipotent stem/precursor cells present in mammalian skin, which may serve as an accessible source of autologous cells for therapeutic applications (Toma et al., 2001).

To date, SKPs have been isolated from hairy skin on the dorsal or ventral trunk of rodents, rodent whisker pad, and adult human scalp, as well as glabrous neonatal and juvenile human foreskin (Toma et al., 2001; Toma et al., 2005; Hunt et al., 2008; Jinno et al., 2010). Lineage tracing experiments indicate that SKPs in facial dermis do indeed arise from the neural crest, whereas the SKPs found in back skin (i.e., dorsal trunk dermis – the cells used in the present work), arise from the somites (Fernandes et al., 2004; Jinno et al., 2010). SKPs isolated from either of those sources, and those isolated from the ventral trunk dermis (presumably of lateral plate origin), all possess highly similar phenotypes according to their differentiation potential and global gene expression (Jinno et al., 2010). Thus, much like mesenchymal
derivatives of the neural crest, SKPs isolated from different anatomical locations arise from
distinct developmental origins, yet converge on to a similar cellular phenotype independently in
each of those tissues (Jinno et al., 2010). What is unusual about the SKPs from the dorsal and
ventral trunk dermis however, is their ability to produce SCs, which were previously only
thought to be made by cells that arise from the neural crest (Jessen and Mirsky, 2005; Dupin et
al., 2006).

Little is known about the niche for SKPs in glabrous skin, but in hairy skin and whisker
pad, SKPs are derived from a Sox2-positive (Sox2⁺) dermal precursor that resides in the dermal
papilla and dermal sheath near the base of hair or whisker follicles (Biernaskie et al., 2009).
Within that niche, those endogenous dermal precursors maintain their multipotency and their
ability to self-renew, and these cells act as a resident dermal stem cell, contributing to hair
follicle morphogenesis, dermal maintenance, and wound healing (Biernaskie et al., 2009). The
contribution to wound healing is of interest here, as Sox2⁺ dermal precursors (i.e., SKPs) were
recruited from hair follicles to contribute differentiated cells to the healing dermis following
punch wound to the skin, and cells from transplanted SKP-derived hair follicles showed the same
behaviour in response to nearby skin injury (Biernaskie et al., 2009). SKPs transplanted into skin
grafts have also been shown to generate SCs that participate in axonal regeneration following
cutaneous nerve injury in vivo (Chen et al., 2012b) and following conditional deletion of the Nf1
(neurofibromatosis type 1) gene, SKPs generate malignant dermal tumours composed of SCs,
melanocytes, and dermal fibroblasts, which are typically found in patients with Nf1 mutations
(Le et al., 2009). On the basis of that work, it has been proposed that SKPs naturally generate
SCs *in vivo* under certain conditions, and thus act as an alternative source of SCs to those that are generated by the dedifferentiation of mature SCs in adult mammalian skin (Chen et al., 2012b).

It is important to point out that SKPs are distinct from other stem cell populations found in adult mammalian skin, including HSCs (Okuno et al., 2002), ASCs (Wolnicka-Glubisz et al., 2005), and hfPS cells (Amoh et al., 2005), all of which express CD34, a marker not generally associated with the Sox2+ dermal precursors that give rise to SKPs under appropriate culture conditions (Biernaskie et al., 2009). SKPs are also distinct from the other CD34-negative stem cells found in the skin, the EPI-NCSCs, as although those cell types share similar molecular properties and differentiation potential, the latter cells are derived from the epidermal bulge region of the outer root sheath of hair follicles, not the dermal papilla/sheath regions where SKPs reside, and they can be readily distinguished based on differential gene expression (Sieber-Blum et al., 2004; Hu et al., 2006; Biernaskie et al., 2009; Hunt et al., 2009; Biernaskie, 2010). Indeed, according to Hu et al. (2006), neither the facial (crest-derived) nor the back skin (non-crest-derived) dermal papilla contains EPI-NCSCs, and the work of Jinno and colleagues (2010) demonstrated that Wnt-1+ neural crest-derived cells isolated from dorsal trunk (i.e., back skin) do not generate SKP-spheres under appropriate conditions *in vitro*.

### 1.8.2 SKPs generate bona fide SCs under appropriate conditions *in vitro and in vivo*

The first evidence that SKPs give rise to SCs came from early experiments examining the differentiation potential of those cells, which demonstrated that when rodent or human SKP spheres are dissociated, plated under conditions that promote their adherence (e.g., in culture dishes coated with poly-δ-lysine [PDL] and laminin), and exposed to serum they generate small numbers of bipolar spindle shaped cells that express characteristic SC markers including GFAP,
CNPase, S100β, and p75 (Toma et al., 2001; Fernandes et al., 2004; Toma et al., 2005; McKenzie et al., 2006). Subsequent work found that exposure to the known SC mitogens, neuregulin and/or forskolin, promoted the differentiation of SKPs into SCs in culture, and those SKP-SCs formed small colonies of bipolar spindle-shaped cells that expressed SC myelin proteins, including PMP-22, MBP, and P₀ in the presence of forskolin (Fernandes et al., 2004; Biernaskie et al., 2006; McKenzie et al., 2006). Furthermore, those colonies could be mechanically isolated using cloning cylinders and replated under the same culture conditions to generate a reasonably pure population of SCs, which could then be expanded and purified even further using sequential passaging, to yield cultures of SKP-SCs that are >95% pure (Biernaskie et al., 2006; McKenzie et al., 2006). While the in vitro evidence of SC differentiation seemed promising, functional testing was clearly required to determine whether the SKP-SCs truly represented a population of SCs analogous to N-SCs.

The seminal work on that topic was conducted by McKenzie and colleagues (2006), who demonstrated for the first time that SKPs represent a source of functional, myelinating SCs for therapeutic application in the injured/dysmyelinated PNS and CNS. Those authors began by demonstrating that both naïve SKPs and SKP-SCs (generated from yellow fluorescent protein [YFP]-positive transgenic mice) transplanted into the injured sciatic nerve of wildtype mice were able to generate mature myelinating SCs that ensheathed axons 1:1 and produced MBP- and PMP-22-positive myelin sheaths. Next they transplanted YFP⁺ SKP-SCs into the crushed sciatic nerve of dysmyelinated shiverer mutant mice (genetically deficient in MBP) and showed that a large number of transplanted SKP-SCs generated MBP⁺ myelin sheaths, whereas a subset of those cells aligned with axons and continued to express GFAP rather than myelin proteins; which
is consistent with a non-myelinating SC phenotype. Using immunoelectron microscopy (immuno-EM), they next demonstrated that those cells produced bona fide MBP⁺ compact myelin with major dense repeating lines, which was structurally indistinguishable from the myelin formed by endogenous SCs in the nerve. And lastly, they used EM to demonstrate that naïve rodent and human SKPs are both able to generate compact myelin in the dysmyelinated brain of newborn shiverer mice.

Thus, in one publication McKenzie et al. (2006) demonstrated that SKP-SCs are able to differentiate into both myelinating and non-myelinating SCs in vivo, that the myelin sheaths generated by SKP-SCs are virtually indistinguishable from those made by endogenous SCs, and that SKP-SCs are able to myelinate both the PNS and CNS axon populations. In light of that work it seems that, unlike any of the other potential sources of autologous SCs described above, the SKP-SCs represent a stable population of bona fide SCs that should provide a suitable alternative to N-SCs for PNS or CNS repair. That being said, it remained to be seen whether the SKP-SCs would provide the same degree of therapeutic benefit in the injured CNS and PNS as N-SCs, which were already well established as an efficacious treatment for SCI and peripheral nerve injury. Furthermore, although the SKP-SCs shared a number of important properties with N-SCs, our knowledge of the precise degree of similarity between cultured SKP-SCs and N-SCs was almost completely lacking, as direct comparisons of those cells either in vitro or in vivo had yet to be conducted.
1.9 Overview of experiments and hypotheses

In this thesis I examine the therapeutic potential of SKP-SCs transplanted into the injured spinal cord using two preclinical animal models, with the goal of establishing the efficacy of those cells as a therapy for SCI. Furthermore, I directly compare those cells to their nerve-derived counterparts both in vitro and following transplantation into the injured spinal cord, in efforts to examine the similarities and differences between SCs generated from SKPs and peripheral nerve, with the goal of determining whether SKP-SCs are truly a suitable replacement for N-SCs as a source of potentially autologous SCs for transplant-based CNS repair.

Chapter 2 of this dissertation describes my initial work assessing the neurobiological and functional outcomes of SKP-SC treatment following delayed transplantation into the contused thoracic spinal cord of the rat. Based on the similarity between SKP-SCs and N-SCs, and the recognized efficacy of N-SCs as a treatment for SCI, I hypothesized that the SKP-SCs would demonstrate efficacy as a treatment for SCI and that they would provide similar benefits to those commonly associated with N-SC transplantation. The results of that work largely supported those hypotheses, as SKP-SC transplantation resulted in greater functional benefits than cellular control transplants (naïve SKPs or neurospheres) and transplanted SKP-SCs exhibited cellular behaviour (e.g., survival, lesion bridging and myelination) and reparative effects (e.g., enhanced tissue sparing and axonal growth) that were highly similar to those typically displayed by transplanted N-SCs. However, despite those similarities, some of the findings from that work differed from previous reports by other groups transplanting N-SCs after SCI. For example, in contrast to the behaviour of N-SCs in most transplantation studies, the SKP-SCs were able to migrate out of the lesion site and enter spared host tissue, where they
appeared to integrate well, myelinating axons in intact tissue adjacent to the lesion site and inducing little astrocyte reactivity in the process. Furthermore, transplanted SKP-SCs facilitated the growth of descending serotonergic and noradrenergic (putative brainstem-spinal) axons into the lesion site, whereas the transplantation of N-SCs into thoracic injury sites is not normally associated with the ingrowth of axon populations in the absence of additional co-treatments. Thus the results of our initial investigation of SKP-SCs as a treatment for SCI suggested that the SKP-SCs have advantages over N-SCs in terms of their suitability and/or capacity for CNS repair.

Having established the efficacy of SKP-SCs as a treatment for thoracic SCI using the clinically relevant contusion model, I next sought to strengthen that position by establishing the efficacy of SKP-SCs as a treatment for cervical injury and by directly comparing the efficacy of SKP-SCs and N-SCs as transplantation-based therapies for SCI. For those purposes, I chose to use a different injury model, as the use of multiple models to establish therapeutic efficacy is desirable due to the extreme heterogeneity of SCI in humans. Chapter 3 of this dissertation describes that work, wherein I examined the neurobiological and functional outcomes of SKP-SC transplantation following a left, cervical dorsolateral funiculus (DLF) crush and compared those results to treatments with N-SCs, skin fibroblasts, or media controls. Based on our earlier findings, I hypothesized that SKP-SC transplantation would demonstrate efficacy as a treatment for incomplete cervical SCI and that those cells would provide enhanced reparative benefits and superior functional recovery compared to their nerve-derived counterparts.
Prior to the completion of that work, I chose to conduct additional comparisons of the SKP-SCs and N-SCs to more closely examine some of the potential differences suggested by our initial study. The enhanced capacity of SKP-SCs to migrate and integrate into intact host tissue might explain the differential regenerative effects of SKP-SCs and N-SCs, as co-treatments that enhance N-SC migration out of the lesion site have also been found to increase the capacity of those cells to facilitate supraspinal regeneration (for example see Ghosh et al., 2012). As such, I focused my efforts on investigating the potential differences between SKP-SCs and N-SCs with respect to their interactions with astrocytes. Given that SCPs are known to migrate through astrocyte-rich territory and intermingle with astrocytes more extensively than N-SCs derived from neonatal tissue (Woodhoo et al., 2007), I speculated that the key difference between SKP-SCs and N-SCs may simply be one of maturity/differentiation. Although the N-SCs typically used for transplantation represent a dedifferentiated SC phenotype that is similar to the immature SCs that arise during development, those phenotypes are not identical, and it may be the case that SKP-SCs represent a less mature/differentiated SC phenotype, by virtue of the fact that they have never fully developed into mature myelinating or non-myelinating SCs. In light of that, I hypothesized that SKP-SCs represent a less mature/differentiated SC phenotype than N-SCs, and I predicted that SKP-SCs would elicit less reactive astrogliosis and display an enhanced capacity for migration/integration astrocyte-rich territory than their nerve-derived counterparts. To address those hypotheses I ran a variety of direct comparisons between neonatal rat SCs cultured from both sources and that work is described here in Chapter 4, which includes examinations of in vitro protein/gene expression and assays assessing the interactions of both cell types with astrocytes in vitro (inverted coverslip migration assay and the boundary assay) and after transplantation into the partially injured cervical spinal cord.
Chapter 2:

Skin-derived Precursors Generate Myelinating Schwann Cells that Promote Remyelination and Functional Recovery after Contusion Spinal Cord Injury


* equal contribution
2.1 Introduction

After injury, the adult mammalian spinal cord exhibits limited endogenous repair and poor functional recovery. Several factors contribute to this, including protracted secondary damage, an inability to remyelinate spared demyelinated axons, and failure of axons to overcome local expression of myelin-associated inhibitory molecules and the reactive astrocytic scar (Deumens et al., 2005; Dietz and Curt, 2006; Harel and Strittmatter, 2006; Yiu and He, 2006). The early pioneering work of Aguayo and colleagues (Richardson et al., 1980; David and Aguayo, 1981) demonstrated that the peripheral nerve environment was conducive for CNS axon regeneration, and subsequent work demonstrated that Schwann cells (SCs) comprised an essential component of this permissive environment. More recently, numerous studies have demonstrated that transplantation of exogenous peripheral nerve SCs can provide both trophic support for spared axons and participate in remyelination of the injured spinal cord (Xu et al., 1997; Keirstead et al., 1999a; Kocsis et al., 2002; Bunge and Pearse, 2003; Pearse et al., 2004a; Oudega and Xu, 2006; Pearse and Barakat, 2006). However, the invasive surgical biopsies required to harvest the nerve, and the difficulties faced in the purification and expansion of SCs from adult nerves (Akiyama et al., 2002) have complicated their clinical application.

Skin-derived precursors (SKPs) are a self-renewing, multipotent precursor that resides within the dermis of both rodents and humans (Toma et al., 2001; Toma et al., 2005). SKPs, which are generated during embryogenesis and persist into adulthood, share characteristics with embryonic neural crest stem cells (Fernandes et al., 2004), including their ability to differentiate into neural crest derived cell types such as peripheral neurons and SCs (Fernandes et al., 2004; Fernandes et al., 2006; McKenzie et al., 2006). In this regard, we recently demonstrated that
SKPs respond to neural crest cues such as neuregulins by generating SCs, and that these SKP-derived SCs (SKP-SCs) express a myelinating phenotype when cocultured with peripheral axons. Moreover, SKP-SCs display similar behaviour in vivo, generating compact myelin when they encounter axons either within the regenerating peripheral nerve or within the dysmyelinated CNS (McKenzie et al., 2006). Because SKPs represent a highly accessible and potentially autologous source of adult precursors that are capable of generating functional SCs, we asked whether naïve SKPs or SKP-SCs could be used to repair the injured spinal cord by remyelinating spared axons, and/or by providing a growth-permissive environment for the regeneration of damaged axons. As a transplant control, we compared naïve SKPs and SKP-SCs to neurospheres derived from the forebrain subventricular zone (SVZ), a well characterized CNS stem cell that is capable of generating oligodendrocytes and that has been reported to provide some benefit after spinal cord injury (Ogawa et al., 2002; Okano et al., 2003; Vroemen et al., 2003; Watanabe et al., 2004; Karimi-Abdolrezaee et al., 2006). Here, we report that SKP-SCs provide a tissue bridge across the site of a contusion lesion, myelinate host axons both in the spared tissue rim and within the transplant site itself, modify the extracellular milieu around the lesion, and ultimately lead to enhanced functional locomotor recovery after spinal cord injury.

2.2 Materials and methods

2.2.1 Animals

Sixty -two adult male Sprague Dawley rats were used in this study. All procedures were approved by the Hospital for Sick Children Research Institute and the University of British Columbia, in accordance with guidelines of the Canadian Council on Animal Care.
2.2.2 Preparation of SKPs and SKP-SCs

Neonatal murine SKPs were cultured as described previously (Toma et al., 2001; Fernandes et al., 2004). Briefly, dorsal back skin from neonatal (postnatal day 0 (P0) to P3) enhanced yellow fluorescent protein (EYFP) mice (The Jackson Laboratory, Bar Harbor, ME) was cut into 2-3 mm² pieces. Tissue was digested with 1 mg/ml collagenase (Type XI; Sigma, St. Louis, MO) for 20-45 min at 37°C, mechanically dissociated and filtered through a 40 μm cell strainer (BD Falcon, Bedford, MA). Cells were plated in Dulbecco’s Modified Eagles Medium (DMEM)/F-12 (3:1; Invitrogen, Carlsbad, CA), containing 1% penicillin/streptomycin (Cambrex, East Rutherford, NJ), 2% B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (EGF), and 40 ng/ml fibroblast growth factor-2 (FGF-2) (both Collaborative Research, Bedford, MA). To generate secondary spheres, SKPs were digested in collagenase (1 mg/ml) for 5 min and mechanically dissociated to single cells and then subcultured at a density of 10-25,000 cells/ml to generate secondary spheres.

Differentiation of SKPs to SCs (SKP-SCs) was done as previously described (Biernaskie et al., 2006; McKenzie et al., 2006). Briefly, primary or secondary spheres were dissociated and plated on 10 cm dishes coated with poly-D-lysine/laminin (BD Biosciences, Bedford, MA), and grown in DMEM/F-12 (3:1) containing 5 μM forskolin (Sigma) and 50 ng/ml neuregulin-1β (R&D Systems, Minneapolis, MN) and supplemented with 1% N2 (Invitrogen). The medium was changed every 3-4 days. Proliferating colonies of SKP-SCs were isolated using cloning cylinders (Corning, Corning, NY), trypsinized from the dish, and replated in the same medium. Cultures of increasing purity were obtained by sequential passaging as cultures reached confluency.
Neonatal neurospheres were generated from the forebrain subventricular zone of the same postnatal EYFP mice that were used for the SKP cultures, and were used as controls for the cell transplantation studies. Specifically, after removal of the back skin, brains of the same mice were carefully dissected to isolate the tissue surrounding the forebrain lateral ventricles. The tissue was briefly digested with 0.1% trypsin, gently dissociated to single cells by trituration, and grown at a concentration of 10,000 cells/ml. Neurospheres were grown under identical conditions to those used for SKPs (DMEM/F12 medium containing FGF-2, EGF, and B27). Floating spheres appeared after 5 days and were passaged every 5 days using trypsin digestion and mechanical dissociation. All neurospheres underwent at least one, and no more than three, passages before transplantation.

2.2.3 Flow cytometry

Before transplantation, flow cytometry was used to assess the percentage of cells staining with the p75 neurotrophin receptor, a marker of SC fate, after differentiation and expansion of SKPs. We also examined the percentage of SKP-SCs maintaining expression of yellow fluorescent protein (YFP). SKP-SC were isolated using cloning cylinders and expanded for 6 weeks in SC differentiation conditions as described previously (McKenzie et al., 2006). SKP-SCs were trypsinized using trypsin-versene (Bio-Whittaker, Walkersville, MD) and resuspended in Hank’s balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS) to inactivate the enzyme. Cells were centrifuged at 1200 rpm for 6 min and resuspended in 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. Next, cells were washed twice by centrifuging at 1200 rpm for 6 min and resuspension in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) (wash buffer). For staining, cells were resuspended
with a polyclonal rabbit anti-p75 neurotrophin receptor (p75NTR) (intracellular domain, 1:1000; Promega, Madison, WI) PBS containing 0.3% triton X-100 and 1% normal goat serum for 30 min. Cells were again washed twice by centrifuging at 1200 rpm for 6 min and resuspended in wash buffer. Staining was visualized using an Alexa 647-conjugated anti-rabbit secondary antibody (1:2000; Invitrogen) in PBS containing 1% BSA. For negative controls, exposure to the primary antibody was omitted. Analysis was done using a FACSCalibur flow cytometer (BD Biosciences), and 10,000 events were collected for each population.

### 2.2.4 Spinal cord contusion injury and cell transplantation

Adult male Sprague Dawley rats (286 ± 7 g; Charles River Laboratories, Wilmington, MA) were acclimatized to the behavioural testing environment (see below) to establish their behavioural baselines. All surgeries were performed under aseptic conditions. Body temperature was maintained at >36°C throughout the surgical and postsurgical period, and blood oxygenation level and heart rate monitored. Rats were anesthetized with a mixture of ketamine hydrochloride (72 mg/kg) and xylazine hydrochloride (9 mg/kg) via intraperitoneal injection. The skin of the back was shaved and disinfected, and the dorsal aspect of the spinal column exposed at the T8-T10 vertebrae. A laminectomy of vertebra T9 was performed using a fine pair of rongeurs and care was taken not to damage the dura. Rats were then transferred to the stage of the Ohio State University spinal cord impactor device and the dorsal process of vertebrae T8 and T10 were held in place with Allen clamps, while 50% of the body weight was supported from underneath. The initial touch point of the impactor with the dura was determined (using the vibrator mode of the impactor tip) and from there a 1.5 mm displacement with 6 ms dwell time was applied to the spinal cord. Force curve readings revealed values with an average of 286 ± 5 kdyn. Slippage or
excessive deviation from these values led to automatic exclusion of the animal from further study (~ 15% in our hands). This type of injury consistently results, in our hands, in complete loss of hindlimb motor function on day 2 (Basso, Beattie, Bresnahan [BBB] score <2), followed by gradual recovery of hindlimb weight supported stepping with no or only occasional forelimb-hindlimb coordination (BBB score ≈ 11). Immediately after the injury and for an additional day, rats received Ringer’s solution for hydration (5ml, s.c.) and buprenorphine (0.3 mg/kg) to alleviate pain. Bladders were expressed three times daily until the rats reached spontaneous micturition. Antibiotics (Baytril, 0.06 ml, i.m.) were given to treat/prevent bladder infection.

For transplantations, 7 days after contusion injury, rats received no treatment (medium injection alone, n=4), or were transplanted with one of three different cell types; neonatal murine SVZ neurospheres (n=11), undifferentiated neonatal SKPs (n=13), or SKP-SCs (n=16). Immediately before transplantation, spheres of SKPs or neurospheres were digested with 1mg/ml collagenase and gently triturated to yield a single-cell suspension. Cells were resuspended at a final concentration of $2 \times 10^5$ cells/µl and a total volume of 5µl was stereotaxically injected into the epicentre of the lesion using a 10µl Hamilton syringe fitted with a glass micropipette. For the purposes of estimating the percent survival of transplanted cells we assumed that only about $8 \times 10^5$ cells (80% of the projected number) managed to actually pass through the injection pipette into the spinal cord; which is an extremely conservative estimate given that Hill et al. (2007) previously reported cell losses of ~40% under similar conditions. All animals received daily subcutaneous injections of cyclosporine A (15 mg/kg s.c.; Novartis, East Hanover, NJ) beginning two days prior to transplantation and continuing for the duration of the experiment. Behavioural testing continued for 8 weeks after transplantation, and the animals were reanaesthetized at 10
weeks after injury (as described above) in order to trace the ascending sensory fibres and
descending corticospinal fibres. To trace sensory fibres, Cholera toxin B (CTB) (1µl; List
Laboratories, Campbell, CA) was injected into the lumbar sciatic nerve at the sciatic notch.
Alternatively, to trace the corticospinal tract, biotinylated dextran amine (BDA) (10%, 4 times
0.5µl; Invitrogen) was injected into the hindlimb area of sensorimotor cortex using a fine glass
capillary attached to a Hamilton Syringe. Injection was controlled by an automatic advancement
device which allowed the tracer to be injected at rates not exceeding 100 nl/min. For ethical
reasons, rats were randomized to receive either CTB or BDA, but not both. Neuroanatomical
tracing procedures precluded additional behavioural testing of the animals.

To quantitatively assess the effect of transplant on axonal growth a subset of animals
were engrafted with either SKP-SCs (n=10) or medium only (n=8) and were killed at 1 or 2
weeks after transplantation. All other rats were killed at 1 or 2 weeks after the tracer injections
(~12 weeks after injury) with a lethal dose of ketamine/xylazine and were then transcardially
perfused with PBS, followed by 4% paraformaldehyde. The spinal cords were dissected,
postfixed overnight in the same fixative, and cryoprotected with 30% sucrose in 0.1 M PBS.

2.2.5 Tissue processing and immunocytochemistry

Immunocytochemistry on cultured cells was performed in plastic chamber slides or
dishes as we described previously (McKenzie et al., 2006). For spinal cords, 20 µm longitudinal
sections were cut on a cryostat in the horizontal plane and mounted on Superfrost Plus slides
(Fisher, Houston, TX). Sections were permeabilized with 0.2% triton X-100 and nonspecific
binding sites were subsequently blocked with 10% normal goat or donkey serum. For
immunodetection of myelin basic protein (MBP) and P0, tissue sections were delipidized before
any staining procedures. Primary antibodies were incubated overnight at 4°C, washed, and then incubated with fluorescent-conjugated secondary antibodies for 2 hours at room temperature. The following primary antibodies were used: anti-βIII-tubulin monoclonal (1:500; Covance, Princeton, NJ), rabbit anti-neurofilament M (NFM; 1:500; Chemicon, Temecula, CA), polyclonal chicken anti-green fluorescent protein (GFP; 1:1000; Chemicon), polyclonal chicken anti-P₀ (1:1000; Aves Labs, Tigard, OR), anti-GFAP polyclonal (1:500; DakoCytomation, Carpinteria, CA), polyclonal anti-p75NTR (1:500; Promega or Chemicon), monoclonal anti-rat p75NTR (1:500; Chemicon), anti-S100β monoclonal (1:1000; Sigma), polyclonal anti-collagen type II (1:200; Chemicon), polyclonal anti-fibronectin (1:400; Sigma), monoclonal anti-neurocan (1:500; Chemicon), monoclonal anti-CTB (1:500; Chemicon), anti-MBP monoclonal (1:200; Chemicon), anti-MBP monoclonal (1:100; Serotec, Oxford, UK), rabbit anti-laminin (1:200; Sigma), polyclonal anti-tyrosine hydroxylase (TH) (1:200; Chemicon), rabbit anti-serotonin transporter (1:500; Immunostar, Hudson, WI), rabbit anti-calcitonin gene-related peptide (CGRP) (1:500; Sigma), polyclonal anti-neuron-glia antigen 2 (NG2; 1:200; Chemicon), anti-Ki67 monoclonal (1:200; BD Pharmingen, San Diego, CA), polyclonal anti-contactin-associated protein (Caspr; a generous gift from Dr. J. Trimmer, University of California, Davis, CA), and anti-Kv1.2 potassium channels (Dr. J. Trimmer). Secondary antibodies used were as follows: Alexa 488-conjugated goat anti-mouse, Alexa 555 goat anti-mouse IgM, Alexa 555 goat anti-rabbit, and Alexa 350 goat anti-mouse (1:1000; all from Invitrogen) and FITC-conjugated, AMCA (7-amino-4-methylcoumarin-3-acetic acid)-conjugated, or Cy3-conjugated donkey anti-mouse, -rabbit, -chick, or -goat (Jackson ImmunoResearch, West Grove, PA). Immunofluorescence was visualized using a Zeiss (Oberkochen, Germany) Axioplan 2 microscope fitted with deconvolution software (Northern Eclipse; Empix, Mississauga, Ontario,
Canada). Colocalization was confirmed by adjacent 1 µm optical sections using a Zeiss LSM 5 confocal laser-scanning microscope.

2.2.6 Quantification of cavity and transplant volume, tissue sparing, cell survival, myelination, and axon numbers

To determine transplant volumes, serial spinal cord sections (200 µm apart) immunostained for YFP were photographed at 10X primary magnification. Using Northern Eclipse Software (Empix), regions containing YFP-expressing cells were outlined and the area was measured. Transplant volumes were determined using the following equation: $V = \sum (\text{area} \times \text{section thickness} \times \text{number of sections in each sampling block})$. Quantification of cavity volumes were determined in an identical manner, using serial photographs of YFP and P0 immunofluorescence. To estimate the amount of spared spinal cord tissue, widths of the thinnest portion of spared tissue around the lesion cavity (i.e. spared host rim) were measured every 200 µm through the extent of each spinal cord. These measurements were then summed, providing a conservative estimate of spared host tissue for each animal.

Survival of transplanted, YFP-positive cells was determined for the five animals with the largest transplant volumes in each group using unbiased stereological techniques. The spinal cord was divided longitudinally into three blocks. One section from each block was sampled such that three randomly selected regions of the transplant from each section were photographed at 40X primary magnification. The number of YFP-positive cells as well as the number of YFP-positive cells generating a P0-positive myelin sheath within each given area were determined, and the mean density (cells/mm³) for each block was calculated. The total number of surviving YFP-positive cells was then estimated by multiplying by the transplant volume.
To quantify endogenous SC remyelination, the spinal cord was again divided into three blocks. One spinal cord section from each block was sampled to determine all of the P₀-positive myelin sheaths within the lesion in that section, versus those within the intact host tissue in the same section. The total number of P₀-positive myelin sheaths within each block was calculated using the following equation: number of P₀ sheaths = Σ(number of cells X number of sections in each block).

To determine the effect of transplantation on axonal sparing, or sprouting/growth, the number of axon fibres found within the lesion at 1, 2 (medium control vs. SKP-SC) and 11 weeks (SKP-SCs vs. SKPs vs. neurospheres) after transplant were counted. Spinal cords were immunostained for TH or 5-hydroxytryptamine (5-HT) to identify a subset of descending axon fibres and these fibres were counted at 200X magnification. Lesion cavity was defined as the areas of tissue containing transplanted (YFP-positive) cells, and/or regions within the lesion cavity that exhibited a loss of GFAP-positive astrocytes (typical of intact host CNS tissue). This is an important consideration particularly with regard to medium treated control animals, who during the first 2 weeks after injury, contain areas rich with immune cells (i.e., macrophages) but few astrocytes, and yet still contain axon fibres that are presumably spared or sprouting. From 20 µm longitudinal sections, every 10th section through the entire thickness of the spinal cord was sampled. Data are presented as group means of actual counts (± SEM) and have not been normalized for the entire cord. Five animals from each group were examined at each time point, with the exception of the 1 week medium control group (n=3). All measurements and counts were done by an observer who was blinded to experimental conditions.
2.2.7  **Behavioural assessment**

Behavioural assessments of locomotor and sensory function were conducted to compare the functional outcome of various treatments over time. Hindlimb locomotor function was assessed by open field locomotion and horizontal ladder paradigms, whereas hindlimb sensory function was assessed using sensitivity tests of mechanical or thermal stimuli. All behavioural tests were conducted 1-2 days before injury to establish baseline values. Open field testing was done again at 6 days after injury and the resulting BBB score was used to eliminate animals that demonstrated a score >10 at that time point to standardize injury severity. The animals were transplanted at 7 days after contusion and were then allowed to rest for 1 week before resuming behavioural testing. Open field testing was conducted on a weekly basis from 2 weeks after injury (i.e., 1 week after transplant) to 9 weeks after injury. All other behavioural tests were delayed until the animals had regained plantar placement with weight support. Specifically, the horizontal ladder test was conducted again at 5 and 9 weeks after injury, whereas the sensory tests were conducted at 5, 7, and 9 weeks after injury.

2.2.7.1  **Open field test**

For open field locomotion testing, the animals were placed in a large Plexiglas open field and observed by two raters (blinded to treatment) for 4 min to assess their performance using the BBB locomotor rating scale (Basso et al., 1995) and the BBB subscore scale (Basso, 2004). Briefly, the BBB is a scale from 0-21 that provides a gross indication of locomotor ability. Scores from 0 to 8 indicate improving degrees of movement of hip, knee, and ankle joints, whereas scores from 9 to 14 indicate various stages of weight support, stepping, and coordination. In our hands, medium treated animals with moderate contusion injuries such as
those used in this experiment commonly plateau at consistent weight supported stepping with no forelimb-hindlimb coordination (BBB score = 11) between 5 and 12 weeks after injury (data from previous work not shown). This scale fails to account for finer details of locomotion (toe clearance, paw position, and tail position, which impact BBB scores from 15-21) until a rat has achieved consistent coordination of weight supported plantar stepping (BBB score = 14). As such, we used the BBB subscore to assess individual components of hindlimb locomotor function, separate from the overall BBB score. The subscore is a scale ranging from 0 to 13 with 0 representing no parallel paw positioning, no toe clearance, and no tail lifts, and 13 representing parallel paw positioning at initial contact and liftoff, consistent toe clearance, and a raised tail during locomotion. All animals included in the present study scored a 21 on the BBB and a 13 on the BBB subscore during baseline assessments. The cage mates that had already completed testing on a given day were occasionally placed in the open field to promote spontaneous locomotion of less cooperative animals, and animals were occasionally given an extra minute in the open field to ensure adequate movement for accurate scoring.

2.2.7.2 Horizontal ladder test

Error (i.e., footslip) frequency was assessed using a horizontal ladder modified as described previously (Metz and Whishaw, 2002). Briefly, rats were videotaped as they walked along a horizontal ladder with variable rung spacing. Subsequent video analysis provided scores for number of steps overall and number of errors (i.e., missteps or footslips) and the total number of errors was divided by the total number of steps to provide an error ratio that was used as the final measure of functional outcome.
2.2.7.3  Mechanical sensitivity test

The threshold of mechanical stimulation for each rat was tested using a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy). Similar to an automated von Frey hair test, this device pushes a thin, semi-rigid, plastic probe through a wire-grated floor and against the plantar surface of the paw from beneath. The probe was set to exert a load increasing at a rate of 5 g/s to a maximum load of 50 g and the device automatically stops and records the force at which the animal withdraws the limb. Each hindlimb was tested twice and the maximum force applied before withdrawal was averaged for all four values as a measure of that animal’s threshold of response to mechanical stimulation in a given testing week. Those values were compared with baseline measurements acquired before injury to determine whether any of the treatments elicited responses indicative of mechanical allodynia (i.e., neuropathic pain).

2.2.7.4  Thermal sensitivity test

This test used an infrared (IR) Plantar Test device (Ugo Basile) based on the Hargreaves method (Hargreaves et al., 1988) to assess latency of withdrawal after exposing the hindlimb plantar surface to a radiant heat source. The IR intensity of the device was set at 50% to ensure that the animals would suffer no tissue damage even at the maximum duration of heat application (set at 30 s). The device shuts off automatically and records the latency when an animal withdraws its foot from the IR beam. On testing days, latency of withdrawal from the IR source was assessed twice for each hindlimb, for a total of four measures, which were averaged to provide the threshold of response to heat stimulation each week. Those values were compared with baseline to determine whether any of the treatments caused thermal hyperalgesia indicative of neuropathic pain in response to heat stimulation.
2.2.8 Statistical analyses

All data are presented as mean ± SEM. Histological data, including cavity volume, cell survival, myelination, and axon counts were analyzed using one-way ANOVA, and group differences were ascertained using Fisher’s PLSD post hoc comparisons. Behavioural data were analyzed using repeated-measures ANOVA and group differences were ascertained using unpaired t-tests. For data that violated the assumptions of normality and/or homogeneity of variance (i.e., von Frey mechanical sensitivity test) of the parametric tests, the equivalent nonparametric tests (Kruskal-Wallis and Mann-Whitney U) were used. The significance level for all tests was set at $p < 0.05$.

2.3 Results

2.3.1 Isolation, expansion and characterization of SKPs and SKP-derived SCs for transplantation

We previously showed that SKPs will efficiently generate myelinating SCs when differentiated in the presence of neuregulins (McKenzie et al., 2006). Moreover, even naive SKPs will generate myelinating SCs when transplanted into the regenerating peripheral nerve (McKenzie et al., 2006). Because SCs have been proposed as a treatment for spinal cord injury, we generated both SKPs and SKP-SCs for transplantation experiments. Specifically, YFP-tagged SKPs were generated from neonatal (postnatal days 1-3) mouse backskin by culturing in FGF-2 and EGF and passaging the floating spheres at two weeks, as described previously (Toma et al., 2001; Fernandes et al., 2004; Biernaskie et al., 2006; McKenzie et al., 2006). These secondary SKP spheres expressed characteristic markers, including nestin, fibronectin, and versican (Fernandes et al., 2004) and were either used directly for transplantation, or were differentiated
into SKP-SCs before transplantation. For these differentiations, SKPs were cultured in the presence of neuregulin-1\(\beta\) and forskolin (Biernaskie et al., 2006; McKenzie et al., 2006). SKP-SCs were identified morphologically, mechanically isolated and then further expanded in the same gliogenic conditions. After three passages, the putative SKP-SCs had expanded significantly, maintaining both their bipolar morphology (Fig. 2.1A) and their expression of the YFP reporter gene (Fig. 2.1B). The purity of these SKP-SC cultures was confirmed by flow cytometry, which demonstrated that \(\geq 97\%\) of the cells were positive for both the YFP reporter, and for the p75NTR (Fig. 2.1C,D), a marker of SCs or neural crest precursors. Moreover, immunostaining confirmed that the YFP-positive SKP-SCs coexpressed S100\(\beta\), p75NTR, and P\(_0\) (Fig. 2.1E,F), an expression profile characteristic of SCs and SC precursors.

As a control for these transplantations, we generated neurospheres from the neonatal forebrain SVZ, because SVZ-derived neurospheres have previously been transplanted into the injured rat spinal cord (Vroemen et al., 2003; Karimi-Abdolrezaee et al., 2006). To obtain these cells, we used brains from the same neonatal, YFP-expressing mice used to generate SKPs. We previously characterized similar cultures and demonstrated that they express appropriate markers and differentiate into neurons, astrocytes and oligodendrocytes (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Craig et al., 1996).

2.3.2 Transplanted SKPs and SKP-derived SCs survive in the contusion lesion cavity and SKP-derived SCs promote sparing of the host spinal cord tissue

To ask whether SKPs or SKP-SCs represent a potential cell source for treatment of spinal cord injury, we contused rats and then transplanted cells into the epicentre of the spinal cord
lesion cavity one week later. Animals were then assessed for sensorimotor function over 9 weeks, and examined neuroanatomically at 12 weeks after injury.

Analysis of longitudinal sections through the contused spinal cord revealed that SKPs, SKP-SCs, and neurospheres demonstrated distinct differences in terms of survival, integration pattern, and host tissue protection. Contusion injury typically results in the formation of a large cavity, leaving only a small rim of spared host tissue, a result that was obtained when medium alone was injected (Fig. 2.2A). Transplanted neurospheres were highly compromised in their survival after transplantation into the lesion cavity, and only small numbers (<15,000) were found scattered along the edge of the lesion cavity (Fig. 2.2B,H), a finding consistent with previous studies showing that neurospheres do not survive well within the lesion site (Ogawa et al., 2002; Okano et al., 2003; Watanabe et al., 2004). However, those few neurosphere cells that entered the spared spinal cord parenchyma survived and displayed some migration (Fig. 2.2B). In contrast to neurospheres, survival of SKPs and SKP-SCs was far more robust, with ~180,000 and 140,000 YFP-positive cells surviving at 11 weeks after transplantation (Fig. 2.2C,D,H). Transplant morphology was, however, dramatically different for SKPs versus SKP-SCs. Although both reduced the degree of cavitation (Fig. 2.2C-E), they differed significantly in their integration into the host spinal cord. SKP-SCs consistently showed good integration into the host spinal cord, extending through the length of the spinal contusion site in a predominantly rostral-caudal alignment. Notably, there was no sharp delineation between the transplant and the host tissue rostral and caudal to the transplant, and many SKP-SCs integrated from the transplant into the spinal cord (Fig. 2.2D). Within the transplant, the YFP-positive SKP-SCs were uniformly spindle-shaped, and were densely packed (Fig. 2.2D). Importantly, the SKP-SCs had a
Figure 2.1 Characterization of SKP-derived SCs before transplantation

(A, B) Photomicrographs of SKP-derived SCs generated from a mouse that expresses YFP in all cell types. Both panels show the same field photographed with phase illumination (A) and with fluorescence illumination (B). (C, D) Flow cytometry with SKP-derived SCs for the genetic YFP tag (C; GFP) and for p75NTR (D; p75), a cell surface marker for SCs. Note that >97% of SKP-derived SCs express both of these proteins. (E) Photomicrographs of SKP-derived SCs triple-labelled for the genetic YFP tag, and the SC markers S100β and p75NTR (p75). The bottom right panel is the merge. The arrows indicate a triple-labelled cell. Note that virtually all of the cells express all three proteins. (F) Photomicrographs of SKP-derived SCs double-labelled for the genetic YFP tag, and the peripheral myelin protein P0. The top left panel is Hoechst nuclear staining to show all of the cells in the field, and the bottom right panel is the merge. The arrows indicate double-labelled cells.
Figure 2.2  Cavity size and cell survival in the contused rat spinal cord after transplantation of SKPs versus SKP-derived SCs

(A-D) Fluorescence, low-magnification (10X) photomicrographs of longitudinal sections through the spinal cord of rats that were transplanted 11 weeks earlier with medium alone (A), YFP-expressing neonatal forebrain SVZ neurospheres (B), YFP-expressing neonatal SKPs (C), or YFP-expressing SKP-derived SCs (D). (E-G) Note that only SKP-derived SCs formed bridges across the longitudinal plane of the cavity with good rostrocaudal integration. Quantification of sections similar to those shown in A-D to obtain the mean cavity size (E), the amount of spared tissue (F), and the transplant size (G). *p<0.05 relative to medium alone (E,F), and/or to neurospheres (F,G). Note that naïve SKPs significantly reduced the cavity size and demonstrated the largest transplant volumes, whereas SKP-derived SCs significantly spared the tissue rim. (H) Number of surviving, YFP-positive cells 12 weeks after transplantation. Note that of the three groups, SKPs had the highest survival, followed by SKP-derived SCs, both showing significantly greater survival than neurospheres. * p< 0.05. In E-H, results represent mean ± SEM.
significant effect on the survival and maintenance of the host tissue surrounding the lesion cavity, leading to significant sparing of the tissue rim relative to both medium alone and neurosphere transplants (Fig. 2.2F); a finding consistent with a neuroprotective effect. In contrast to SKP-SCs, although transplanted SKPs filled much of the lesion cavity (Fig. 2.2C,E,G), there was a sharp demarcation between the transplant and the host spinal cord tissue, and the transplanted cells did not display any predominant orientation (Fig. 2.2C). Within the transplant, the SKPs differentiated into a variety of cell shapes, indicative of different cell types (described below), and they were packed much more loosely than the SKP-SCs. Immunostaining for the proliferation marker Ki67 indicated that, at this late time point after transplantation, very few donor YFP-positive SKPs were proliferating (<2%) (Fig. 2.3D). Thus, both SKPs and SKP-SCs survive well in the lesioned spinal cord, but only SKP-SCs provide significant sparing of host tissue surrounding the lesion.

2.3.3 Transplanted SKP-derived SCs modify the extracellular environment after spinal cord lesion

The observed differences in transplant morphology suggested that there were differences in integration of the transplanted cells with the host tissue, and perhaps in reactive gliosis. To address this, we performed immunocytochemical analysis for YFP (to identify transplanted cells), GFAP (to assess reactive gliosis), and rat p75NTR (to identify endogenous SCs); host astrocytes express GFAP, but not YFP or rat p75NTR, whereas cells that express rat p75NTR but not YFP will be host SCs. With regard to SKP-SC transplants, there was extensive intermingling between the transplanted cells and host astrocytes, both within the transplant and within the host tissue around the lesion (Fig. 2.4A). There was also extensive intermingling
Figure 2.3 Naïve SKPs are multipotent in vivo, and generate inappropriate cell types within the injured spinal cord

(A-C) Transplants similar to those shown in Figures 2.1-2.9 were immunostained for YFP (green) and for several markers of mesodermal cell types. (A) Transplanted YFP-positive SKPs expressed fibronectin (red, arrows) which in some cases protruded into the surrounding host rim. Note that SKPs were negative for GFAP (blue). (B) Transplanted YFP-positive cells expressed collagen type II (red, arrows), a marker for chondrocytes and dermal fibroblasts. The section was also stained for neurofilament-M (blue). (C) Histological analysis revealed that transplanted SKPs generated cells with a morphology typical of adipocytes (arrows). (D) A small percentage of naïve SKPs still proliferate at 11 weeks after transplantation. Sections were double-stained with YFP for the transplanted SKPs, and with Ki67, a marker for proliferating cells. Note the colocalization of Ki67 in a small percentage of YFP-positive cells (arrows).
between transplanted SKP-SCs and host p75NTR-positive SCs (Fig. 2.4A) (described below). In contrast to the minimal astrocytic response to SKP-SCs, the naive SKP transplants were surrounded by a sharp border of reactive astrocytes expressing high levels of GFAP (Fig. 2.4C), and virtually none of the host astrocytes infiltrated into the body of the SKP transplants. This border of reactive astrocytes was interrupted by groups of invading host rat p75NTR-positive SCs (Fig. 2.4C), which also infiltrated into the transplant itself (described below). A similar GFAP-positive, reactive astrocyte response was seen after transplantation of neurospheres, where the reactive astrocytes lined the walls of the large cavities found in these spinal cords (Fig. 2.4E).

One of the consequences of the reactive astrocyte response after spinal cord injury is increased expression of chondroitin sulfate proteoglycans (CSPGs) such as neurocan. Expression of CSPGs is considered to be one of the negative sequelae of spinal cord injury, because they inhibit axonal growth, and contribute to the barrier imposed by the astrocytic scar (Fitch and Silver, 1997; Bradbury et al., 2002; Borisoff et al., 2003; Jones et al., 2003; Monnier et al., 2003; Tang et al., 2003). Given that transplantation of SKP-SCs caused only minimal reactive gliosis (Fig. 2.4A), we asked whether they also had an impact on expression of extracellular matrix molecules such as neurocan. To do this, we performed immunocytochemistry for YFP (to follow transplanted cells), neurocan, and laminin, the latter of which is a permissive substrate for axonal growth (Grimpe and Silver, 2002). Remarkably, levels of neurocan in the host tissue surrounding the SKP-SC transplants, and in the transplants themselves, were very low, similar to levels seen in uninjured regions of the spinal cord (Fig. 2.4B). In contrast, as predicted by the reactive gliosis seen around the SKP transplants, there was a pronounced increase in neurocan expression in the host spinal cord adjacent to and rostral to SKP transplants (Fig. 2.4D). A distinct increase in
Figure 2.4 Transplanted SKPs and SKP-derived SCs modify the extracellular environment surrounding the lesion

(A-F) Fluorescence photomicrographs of longitudinal sections through the spinal cord of rats that were transplanted 11 weeks earlier with neonatal YFP-expressing SKP-derived SCs (A,B), YFP-expressing SKPs (C,D), or YFP-expressing forebrain SVZ neurospheres (NS) (E,F), showing both the transplant and the adjacent tissue rim. (A,C,E) Immunocytochemistry for YFP expressed by the transplanted cells (green), for rat-specific p75 neurotrophin receptor (red), which is specific for host SCs, and for GFAP (blue) which, in the absence of coexpression of p75NTR, is specific for astrocytes. The Arrowheads denote GFAP-positive astrocytes, and the arrows denote host SCs. Note that SKP-derived SCs (green) allow infiltration into the transplant of both astrocytes (blue) and SCs (red) from the host tissue. The same occurs, albeit to a significantly lesser extent, for transplanted SKPs. (B,D,F) Immunocytochemistry for YFP expressed by the transplanted cells (green), for neurocan (red), an inhibitory ECM molecule, and for laminin (blue). The arrows and arrowheads denote regions of neurocan and laminin expression, respectively. Note that the expression of neurocan (red) is greatly reduced in the spinal cord tissue surrounding the SKP-derived SC transplant relative to transplants of either SKPs or neurospheres. Moreover, laminin (blue) is expressed throughout the transplant and to some extent in the intact rim in spinal cords receiving SKP-derived SCs or SKPs, but is only found in the border zone lining the mostly empty cavities of neurosphere transplants.
neurocan was also seen around the lesion cavities of the neurosphere-transplanted animals (Fig. 2.4F). Differences were also observed in the laminin response between these different groups. The SKP-SC transplants displayed abundant laminin immunoreactivity within the transplant and in the surrounding host tissue. In SKP-treated spinal cords, laminin was particularly enriched in the borders of the SKP transplants whereas only the walls of the lesion cavities contained laminin in neurosphere transplants. Thus, transplantation of SKP-SCs, but not of SKPs or neurospheres, had very positive effects on the environment of the host spinal cord, suppressing reactive gliosis and neurocan expression.

2.3.4 **SKP-derived SCs promote axonal growth and sprouting into the transplant region**

SCs, either alone or in conjunction with other therapies, have previously been shown to provide a conducive environment for the growth and regeneration of CNS axons after spinal cord injury (Paino and Bunge, 1991; Paino et al., 1994; Xu et al., 1995a; Xu et al., 1997; Tuszynski et al., 1998; Weidner et al., 1999; Xu et al., 1999; Azanchi et al., 2004). We therefore characterized axonal growth in the transplant groups, analyzing both ascending and descending fibres.

Immunocytochemical analysis of the 12 week transplant group for neurofilament and tubulin revealed that SKP-SCs were regularly associated with very large numbers of axons both adjacent to and within the transplants (Fig. 2.5; or for examples, see Figs. 2.9C,H and 2.11A). Many of these axons traversed the entire injury site in a rostrocaudal orientation, indicating either axonal preservation and/or directed growth of sprouting/regenerating axons. We then analyzed defined subsets of axons by immunocytochemistry for TH (a marker for descending noradrenergic and dopaminergic axons as well as sympathetic fibres), serotonin (5-HT, a marker for descending serotonergic axons) and CGRP (a marker for ascending sensory fibres).
Figure 2.5 SKP-derived SCs support robust axonal growth into the transplant

Spinal cord sections of animals transplanted 11 weeks earlier with SKP-SCs (A) or SKPs (B) were immunostained for YFP to follow transplanted cells, and with antibodies specific for neurofilament-M (NFM) (red). Note that the number of preserved or regenerating axons (arrows) was much greater in the SKP-SC versus SKP transplants.
Figure 2.6 SKP-SCs promote axonal growth and sprouting into the transplant region

(A-K) Fluorescence photomicrographs of longitudinal sections through the spinal cord of rats that were transplanted 11 weeks earlier with neonatal, YFP-expressing SKP-derived SCs (A-D, H), neonatal, YFP-expressing SKPS (E-G, I,J) or neonatal, YFP-expressing forebrain SVZ neurospheres (NS) (K). In all cases, sections were immunostained for GFAP (blue). (A,B) Immunocytochemistry for the YFP tag in the transplanted SKP-derived SCs (green) and for tyrosine hydroxylase (TH, red), a marker for descending noradrenergic fibres. (B) is a higher magnification image, and the arrows indicate TH-positive axons coursing through the SKP-derived SC bridge in the centre of the contusion lesion. (C,D) Immunocytochemistry for the YFP tag in the transplanted SKP-derived SCs (green) and either serotonin (C; 5-HT, red), a marker for descending serotonergic axons, or CGRP (D; red), a marker for sensory axons. The arrows indicate serotonin or CGRP-positive fibres. (E-G) Immunocytochemistry for the YFP tag in transplanted SKPs (green), and for tyrosine hydroxylase (E), serotonin (F; 5-HT), or CGRP (G) (red in all cases). (F) is a higher magnification image. The arrows denote positive axons. Note that, unlike SKP-derived SCs, the axons do not course through the transplant, but instead are primarily limited to the border of the transplant. (H-K) Ascending sensory axons were traced with the anterograde tracer cholera toxin B (CTB) at 10 weeks after lesion, animals were killed 2 weeks later, and immunocytochemistry was performed for the YFP tag in the transplanted cells (green), and for cholera toxin B (red). Photomicrographs of longitudinal sections through the spinal cords of these animals demonstrate that ascending, CTB-positive axons grew at least 1 mm into transplants of SKP-derived SCs (H), but not into transplants of naïve SKPs (I,J) or of neurospheres (K). The arrows in H,I,J indicate the CTB-positive axons, and those in K indicate terminal bulbs of CTB-positive axons.
addition, ascending sensory fibres were traced using CTB, which had been injected into the sciatic nerve 1 week before killing. These analyses (Fig. 2.6A-D,H) supported the notion that SKP-SCs promoted new axonal sprouting and/or regeneration. Specifically, we observed massive infiltration of TH-positive (Fig. 2.6A,B) and 5-HT-positive (Fig. 2.6C) axonal sprouts at the rostral spinal cord/transplant interface, and were able to follow these sprouts as far as 1 mm distally. These axons apparently originated from straight descending projections indicative of their brainstem origin and were associated with the cellular bridges formed by the transplanted SKP-SCs. Similarly, CGRP-positive sensory axons were found growing in close association with the transplanted SKP-SCs, infiltrating and extending through the lesion site in primarily a rostrocaudal orientation (Fig. 2.6D). Moreover, tracing of the ascending sensory axons by immunocytochemistry for CTB revealed that these axons grew >1 mm into the SKP-SC transplants (Fig. 2.6H; corresponding image of only CTB-labelled axons is provided in Fig. 2.7C). Thus, SKP-SCs provide an environment that is highly conducive to the maintenance and/or growth of axons.

Very different results were obtained when transplants of naive SKPs were analyzed in a similar manner. Immunocytochemical analysis for NFM and βIII-tubulin indicated the presence of many fewer axons within the SKP transplants relative to the SKP-SC transplants (Fig. 2.5B; for examples, see Figs. 2.9E, 2.11D,E). Immunocytochemical analysis for TH, 5-HT and CGRP revealed that axons positive for these markers were primarily found along the host/transplant interface, with only a few extending >500 µm into the transplant (Fig. 2.6E-G). Of those axons that did sprout into the transplant, they were somewhat randomly oriented, presumably as a consequence of the relatively random orientation of the differentiated SKP-derived cells within
**Figure 2.7 Transplanted SKP-derived SCs enhance growth/sprouting of ascending sensory fibres**

(A) Low magnification image of the injured spinal cord, 2 weeks after transplant, showing CTB-labelled axons (red; arrowheads) within the lesion cavity surrounded by SKP-SCs. Higher magnification of inset is shown at right. Note the presence of CTB-positive fibres (red) and an absence of GFAP-positive astrocytes (blue) within the graft. (B) Low resolution image of CTB-labelled axons within a SKP-derived SC graft 11 weeks after transplant. High magnification of inset (shown at right) shows CTB-labelled axons (red) within a SKP-derived SC graft (green). Similar to the 5-HT and TH-labelled axons, it appeared that numbers of CTB axons (arrowheads; inset shown at right) were elevated after SKP-SC transplant relative to treatment with neurospheres or naïve SKPs. (C) High resolution image demonstrating extensive numbers of CTB-labelled axons within a SKP-SC transplant. Corresponding image showing GFP-labelled SKP-derived SC transplant is shown in Figure 2.6H.
the transplant region. Analysis of the ascending sensory axons by immunocytochemistry for CTB revealed that these axons failed to enter the naive SKP transplants, and instead looped around and grew along the graft-host interface (Fig. 2.6J). They typically ended in large terminal bulbs indicative of frustrated regeneration (Fig. 2.6I). Thus, SKP transplants promote limited axonal growth relative to transplanted SKP-SCs. Importantly, transplanted YFP-positive SKPs were never found to be positive for any of the neuronal markers that were analyzed (TH, 5-HT, βIII tubulin, or NFM) indicating that naive SKPs did not differentiate into neurons within the injured spinal cord.

In contrast to both SKP-SCs and SKPs, neurospheres did not promote axonal growth, and only limited sprouting of CTB-positive axons was seen in the vicinity of the neurosphere transplants (Fig. 2.6K), likely because of the limited survival of these cells within the lesion site.

Although these data indicate that SKP-SCs provide a supportive axonal environment, they do not address the question of whether SKP-SCs actually enhance sprouting/regeneration of axons, as opposed to merely promoting maintenance of spared axons. To distinguish these two possibilities, we quantified the number of TH and 5-HT-positive axons present within SKP-SC transplants at 1, 2 and 11 weeks after transplant. One week after transplant, SKP-SC transplants contained ~500 and 400 TH- and 5-HT-positive axons, respectively, numbers that were statistically similar to contused animals that were injected with medium alone (Fig. 2.8D,F). At two weeks, medium alone animals did not exhibit a change in axon numbers from one week, and the axons remained primarily within astrocyte-rich regions of intact surrounding tissues (Fig. 2.8A). However, at two weeks, the TH- and 5-HT-positive axons in the SKP-SC grafts (Fig. 2.8B,C) showed a significant increase in number compared with one week after injury by
**Figure 2.8 SKP-SCs promote robust axonal growth in the injured spinal cord**

(A) Top panel shows 5-HT immunolabelled axons 2 weeks after medium-only injection into the contusion site. 5-HT-positive axon fibres (red; arrowheads) are mostly limited to the areas of intact CNS tissue rich with GFAP-positive astrocytes (blue; bottom panel). (B) One week after SKP-derived SCs transplant, 5-HT positive axons (top panel) are primarily limited to intact host tissue (GFAP, blue; bottom panel). (C) In contrast, after 2 weeks many more 5-HT-positive axons (top panel; arrowheads) are observed within SKP-derived SC transplants, likely an indication of axonal preservation or enhanced sprouting. Note that 5-HT fibres are observed even in areas that do not contain GFAP-positive astrocytes (blue; bottom panel). (D) Quantification of 5-HT-positive axons within transplants of SKP-SCs at 1 and 2 weeks after injury. No differences in axon number were observed after 1 week; however, by 2 weeks after transplant, SKP-derived SCs supported a twofold increase in axon number relative to medium-alone controls. *p<0.05. (E) Quantification of 5-HT-positive axons in transplants of neurospheres, SKPs, or SKP-derived SCs (as shown in Fig. 2.6) 11 weeks after transplantation. Treatment with SKP-derived SCs resulted in a fourfold increase in 5-HT axon numbers relative to both SKPs and neurospheres. **p<0.001. (F) Quantification of TH-positive axons within the transplants of SKP-derived SCs at 1 and 2 weeks after injury. There was no difference in TH-positive axon numbers after 1 week; however, by 2 weeks after transplant, SKP-derived SCs supported a threefold increase compared with medium-alone controls and a significant increase compared with SKP-derived SC transplants at 1 week. *p<0.05. (G) Quantification of TH-positive axons in transplants of neurospheres, SKPs or SKP-derived SCs (as shown in Fig. 2.6) 11 weeks after transplantation. After 11 weeks, SKP-derived SC transplants supported a threefold increase in axon number.
relative to SKP- or neurosphere-treated animals. **p<0.01. All group analyses were n=5 with the exception of 1 week media controls (n=3). All data are group means ± SEM.
approximately twofold (TH, $t_{(10)} = 2.93, p < 0.02$; 5-HT, $t_{(10)} = 4.62, p < 0.05$). Remarkably, by 11 weeks after transplant, the number of TH- and 5-HT-positive axons in the SKP-SC transplants had increased another threefold to fourfold to $\sim 3000$ in both cases (TH, $t_{(8)} = 2.744, p < 0.03$; 5-HT, $t_{(8)} = 4.62, p < 0.002$) (Fig. 2.8E,G). In contrast, the neurosphere-treated animals contained only $\sim 500$ TH- and 300 5-HT-positive axons, whereas the SKP transplants contained $\sim 1000$ TH- and 300 5-HT-positive axons (Fig. 2.8E,G). ANOVA revealed a significant effect of treatment such that both TH ($F_{(2, 12)} = 9.618, p < 0.004$) and 5-HT ($F_{(2, 12)} = 29.08, p < 0.0001$) axons were significantly reduced in spinal cords from neurosphere- or SKP-treated compared with SKP-derived SC-treated animals ($p < 0.001$). Although not quantified, CTB-labelled ascending axons exhibited a similar increase at 2 and 11 weeks after treatment with SKP-SC relative to medium alone, neurospheres, or naïve SKP (shown in Fig. 2.7A-C). This dramatic increase in the number of axons in SKP-SC transplants between 1 and 11 weeks after transplant indicates that SKP-SCs enhanced the sprouting and/or regeneration of spinal cord axons, and that these axons grew in a directed rostral-caudal manner through the lesion site.

2.3.5  Naïve SKPs and SKP-derived SCs myelinate axons in the injured spinal cord

One strategy for spinal cord repair is to promote the myelination of demyelinated, spared axons in the lesion rim, and to myelinate any axons that do sprout or regenerate. We therefore asked whether SKP-SCs or naïve SKPs myelinated axons in the injured spinal cord, as they do in the regenerating peripheral nerve (McKenzie et al., 2006). To answer this question, we performed immunocytochemistry for YFP, and for P0, a myelin protein that is specific for SCs and SC-derived myelin. Alternatively, we used an antibody for MBP, another myelin protein expressed in both central and peripheral myelin. This analysis revealed the presence of many
Figure 2.9 Naïve SKPs and SKP-derived SCs myelinate axons in the injured spinal cord.

Analysis of longitudinal sections of the spinal cord of animals transplanted with neonatal, YFP-expressing SKPs (SKP), SKP-derived SCs (SC), or neurospheres (NS) 11 weeks earlier. (A,B) Double-label immunocytochemistry for the YFP tag in transplanted SKPs (green) and for the myelin protein MBP (red; arrows indicate double-labelled cells). B is a higher magnification confocal image showing the bipolar morphology of the YFP-positive, MBP-positive cells. (C) Triple-label immunocytochemistry for the YFP tag in transplanted SKP-SCs (green), for P₀ (red) and for the axonal marker NFM (blue; arrows indicate double-labelled P₀-positive SKP-SCs). The inset shows a high magnification confocal image of the boxed area demonstrating a YFP-positive, P₀-positive SC that is associated with an endogenous axon within the transplant. (D) Low (left panel) and high (right panel) magnification confocal micrographs of a section double-labelled for the YFP tag in the transplanted SKP-SCs (green), and for the SC-specific myelin protein P₀ (red). Note that in the spared rim of the contused spinal cord (the boxed area in the left panel), there are P₀-positive myelin sheaths. At least some of these patches of SC myelin derive from YFP-positive transplanted SKP-SCs, as shown in the right panel (arrows). (E) Triple-label immunocytochemistry for the YFP tag in transplanted SKPs (green), for P₀ (red) and for NFM (blue). The arrows denote transplanted, P₀-positive cells. (F) Transplanted neonatal forebrain SVZ neurospheres also occasionally myelinated host axons (arrows), as indicated by triple-labelling for the YFP tag (green), the myelin specific protein MBP (red) and the axonal marker NFM (blue). (G,H) YFP-tagged SKPs (G) and SKP-SCs (H) induced nodes of Ranvier when they formed myelin sheaths on endogenous axons, as indicated by immunostaining for the paranodal, axonal potassium channel Kv1.2 (red; G; arrows) or contactin-associated protein Caspr (red; H; arrows). Quantification of the number (I) and percentage (J) of surviving, YFP-
positive transplanted cells that coexpressed the peripheral myelin-specific protein P₀ and associated with endogenous axons. Note that ~35 and 15% of SKP-SCs and SKPs, respectively, made P₀-positive myelin sheaths. *p<0.05. Data represent mean ± SEM.
myelinating, YFP-positive SCs within transplants of both SKP-SCs and naive SKPs (Fig. 2.9). For both types of transplants, these YFP-positive, P0-positive, or MBP-positive cells displayed a bipolar morphology (Fig. 2.9A,B), consistent with a SC phenotype, and they were closely associated with and aligned along axons (Fig. 2.9C). In the case of SKP-SC transplants, these SCs were present throughout the lesion tissue bridge and were oriented longitudinally along the axis of the axon tracts that coursed through the transplants (Fig. 2.9C). SKP-SCs also migrated into the spared tissue rim around the lesion, where they myelinated spared host axons that were presumably demyelinated as a consequence of the injury (Fig. 2.9D). In contrast, in SKP transplants, the transplant-derived SCs were more frequently seen at the edges of the transplant, where they were randomly oriented (Fig. 2.9A,B,E). This localization is likely a consequence of the location and random orientation of the host axons within SKP transplants (Fig. 2.6); these axons are likely required to induce the naive SKPs to differentiate into SCs (McKenzie et al., 2006). Transplant-derived myelinating SCs were not observed in the host tissue around SKP transplants. With regard to neurospheres, myelination of spared host axons, presumably by transplant-derived oligodendrocytes, was observed only very occasionally (Fig. 2.9F).

To demonstrate that the transplant-derived P0-positive, MBP-positive SCs were making bona fide compact myelin, we performed immunocytochemistry for the axonal proteins Kv1.2 and Caspr, both of which localize to the Nodes of Ranvier on myelinated axons (Rasband et al., 1998; Rasband and Trimmer, 2001b, a). This analysis revealed that axons associated with YFP-positive myelinating SCs demonstrated appropriate clustering of these two paranodal proteins in both SKP and SKP-SC transplants (Fig. 2.9G,H).
To quantify the number of myelinating cells generated by transplants of SKPs versus SKP-SCs, we counted the number of YFP-positive cells that generated a P₀-positive myelin sheath (for examples of what was counted, see Fig. 2.9C, right; D, right). These counts demonstrated that spinal cords transplanted with SKP-SCs and SKPs contained ~30,000-35,000 and 15,000 transplant-derived myelinating SCs, respectively (Fig. 2.9J). This represented ~35 and 15% of the total YFP-positive cells within these transplants (Fig. 2.9I). Thus, a large percentage of SKP-SCs myelinated both spared and sprouting or potentially regenerating host axons. Interestingly, host axons also apparently provided the cues necessary to induce a subset of naive SKPs to differentiate into myelinating SCs, a phenomenon we observed previously (McKenzie et al., 2006).

This quantification also indicated that the large majority of naive SKPs did not differentiate into a myelinating SC phenotype, and our analysis with NFM/tubulin indicated that they also do not differentiate into neurons. We therefore asked whether they differentiated into mesodermal phenotypes within the environment of the injured spinal cord, performing immunocytochemistry for smooth muscle actin and collagen type II, both mesodermal markers. We also examined the transplants morphologically for adipocytes, a cell type SKPs are known to generate (Toma et al., 2001). This analysis revealed that naïve SKPs generated both smooth muscle actin- and collagen type II-positive cells, as well as cells with the morphology of adipocytes (Fig. 2.3A-C). In contrast, transplanted SKP-SCs were never observed to express any inappropriate markers, and all of the cells maintained an appropriate SC morphology with many expressing p75 (Fig. 2.10) and/or P₀, but not detectable levels of GFAP or S100β.
Figure 2.10 Transplanted, non-myelinating SKP-derived SCs express high levels of p75NTR

Spinal cord sections of animals transplanted with SKP-derived SCs 11 weeks earlier were immunostained for YFP to follow transplanted cells (green) and with an antibody that recognizes both mouse and rat p75NTR (red). SKP-derived SCs that do not elaborate a P_0-positive myelin sheath express high levels of p75NTR (arrows), as well as low levels of P_0 (data not shown).
2.3.6 SKPs and SKP-derived SCs promote recruitment of endogenous, myelinating SCs into the injured spinal cord

During our analysis of transplant-derived myelinating SCs, it became evident that SKP and SKP-SC transplants were promoting robust recruitment of endogenous SCs, because we observed many YFP-negative, P₀-positive cells. We therefore analyzed this recruitment of endogenous SCs, which has been previously described to be enhanced by transplants after spinal cord injury (Ramer et al., 2004c; Hill et al., 2006). Analysis of SKP-SC transplants revealed that both the body of the transplant and the spared tissue rim contained many YFP-negative, P₀-positive myelin sheaths associated with endogenous host axons (Fig. 2.11A). A similar pattern of immunostaining was observed when sections were immunostained for rat-specific p75NTR, which recognizes host, but not transplanted SCs (Fig. 2.11B,C). YFP-negative, P₀-positive myelin sheaths were also encountered in both the SKP transplants and in the neurosphere transplants. In the case of SKP transplants, these endogenous SCs were primarily limited to the exterior part of the transplants, and were not as obvious in the tissue rim (Fig. 2.11D-F). Similar results were obtained in the case of the neurospheres and after medium control injections, although the density of host SCs appeared much lower (neurospheres shown in Fig. 2.9G-I).

To more quantitatively assess this recruitment, we counted YFP-negative cells that displayed a P₀-positive myelin sheath. This analysis revealed that of the myelinating SCs in the spinal cords of SKP and SKP-SC transplanted animals, ~80 and 53% of the cells were YFP-negative, respectively, and thus were presumably recruited from the host tissue (Fig. 2.11J). When these numbers were analyzed with regard to endogenous SCs in the contusion lesion versus the spared rim, it became apparent that equal numbers of endogenous myelinating SCs
Figure 2.11 SKPs and SKP-derived SCs promote recruitment of endogenous SCs into the injured spinal cord.

Analysis of longitudinal sections of the spinal cord of animals transplanted with neonatal YFP-tagged SKPs, SKP-derived SCs (SC) or forebrain SVZ neurospheres (NS) 11 weeks earlier. (A-C) Fluorescence photomicrographs of spinal cord sections immunolabelled for the YFP tag in transplanted SKP-SCs (green), for GFAP (blue), and for either P₀ (A) or rat p75NTR (B,C) (both red). C is a higher magnification image. (D-F) Immunolabelling of SKP transplants for the YFP tag (green), the axonal protein NFM (NF; blue), and either P₀ (D) or p75NTR (F). (G-I) Immunolabelling of neurosphere transplants for the YFP tag (green), GFAP (G,I) or NFM (H) (both blue), and rat p75NTR (G,I) or P₀ (H) (both red). In all panels arrows denote host SCs that are YFP-negative and P₀-positive or YFP-negative and rat p75NTR positive. (J) Quantification of the percentage of P₀-positive myelin sheaths double-labelled for the YFP tag present in transplanted SKPs or SKP-derived SCs. The other P₀-positive myelin sheaths derive from endogenous SCs that have migrated into the injured spinal cord. (K,L) Quantification of the total number of P₀-positive myelin sheaths within the lesion site (K) and within the spared rim tissue (L) 11 weeks after transplantation with neurospheres, SKPs, SKP-derived SCs, or medium alone. Note that both SKPs and SKP-derived SCs recruit substantially more endogenous SCs than do transplants of neurospheres or medium alone. * p<0.05; ** p<0.05 relative to both medium control and neurosphere-treated animals. In J-L, results represent mean ± SEM.
were present in the lesion cavities transplanted with SKPs versus SKP-SCs (~60,000 per spinal cord in both cases) (Fig. 2.11L), but that SKP-SC transplants recruited significantly more endogenous myelinating SCs into the spared tissue rim (~30,000 cells per spinal cord) (Fig. 2.11L). A similar analysis of the neurosphere transplants and the medium alone group revealed that endogenous SCs were recruited in both cases, but that the numbers were significantly lower than for transplants of SKPs and SKP-SCs (Fig. 2.11K,L). Thus, not only do transplanted SKP-SCs and SKPs directly generate myelinating SCs but they also recruit endogenous myelinating SCs both into the lesion cavity and into the spared tissue rim.

### 2.3.7 SKP-derived SCs promote functional improvement after contusion injury

These results indicated that SKP-derived SCs promoted tissue sparing, remyelination and axonal growth after contusion injury. We therefore asked whether they also promoted functional locomotor recovery. To address this question, locomotor function was tested before the injury, and then at regular intervals up to 9 weeks after injury. After contusion injury, all transplanted animals displayed gross motor impairment showing only joint movement for the first couple of days and no weight supported stepping for the first week (BBB score < 10), and partial improvement over the ensuing 9 weeks after injury (Fig. 2.12A). Seven weeks after injury, most animals had regained hindlimb stepping without forelimb/hindlimb coordination. However, unlike naïve SKP- or neurosphere-transplanted animals, which appeared to plateau at 7 weeks, the animals transplanted with SKP-SCs showed additional improvement, many exhibiting coordinated forelimb/hindlimb stepping; a critical signpost of recovery from spinal cord injury. Indeed, 6 of 13 animals treated with SKP-SCs exhibited consistent stepping with at least occasional forelimb/hindlimb coordination (BBB score ≥ 12), whereas only 2 of 11 and 4 of 12
Figure 2.12 SKP-derived SCs improve locomotor function after a contusion injury of the spinal cord.

(A) Locomotor function 9 weeks after injury, and 8 weeks after transplantation of neonatal SKPs (n=13), SKP-derived SCs (n=16), or forebrain SVZ neurospheres (n=11), as assessed by the BBB. Although all groups showed gradual improvement over the 9 weeks after injury, group comparisons showed that SKP-derived SCs showed a small but significant behavioural improvement relative to the other groups. (B) The Basso locomotor subscore from the animals shown in A. SKP-derived SCs led to significantly enhanced locomotor behaviour relative to transplants of either naïve SKPs or neurospheres. (C) Locomotor activity in the same group of animals as assessed by ladder rung walking. Animals receiving transplants of SKP-derived SCs showed a reduced number of hindlimb stepping and forelimb placement errors relative to those with transplants of SKPs or neurospheres, but this did not reach significance (p>0.10). In all panels, results represent mean ± SEM. * p<0.05 relative to SKPs; ** p<0.05 relative to all other groups. Group differences were ascertained using unpaired t-tests.
animals reached this level of functionality after treatment with neurospheres or SKP, respectively. At week 9 after injury, post hoc tests revealed a modest, but significant improvement in gross motor performance in animals receiving SKP-SCs relative to naïve SKPs ($t_{(27)}=1.852$, $p<0.04$; Fig. 2.12A), but there was no statistical difference between SKP-derived SCs and neurosphere-treated animals.

To further examine specific locomotor components (i.e., paw position, toe clearance, trunk control, and tail position) we used the Basso subsoring scale (Basso, 2004). Repeated measures ANOVA revealed a significant interaction of treatment and time ($F_{(6, 32)}= 8.10$, $p<0.000$). In parallel with the gross motor measure, animals treated with SKP-SCs began to show persistent functional gains between 7 and 9 weeks after injury, which was not observed in either SKP- or neurosphere-transplanted animals (Fig. 2.12B). Within the SKP-SC group, 8 of 14 animals achieved locomotor subscores of $\geq 5$, whereas only 3 of 11 neurosphere-transplanted and 2 of 14 SKP-transplanted animals achieved this level of motor function. At 9 weeks after injury, ANOVA ($F_{(2,37)} = 5.14$, $p<0.02$) and group comparisons confirmed that SKP-SC motor performance was significantly improved relative to both SKP- (p<0.005) and neurosphere-treated (p<0.05) groups. In addition, we assessed hindlimb placing and stepping errors in the horizontal ladder test (Metz and Whishaw, 2002). Although the SKP-SC group made fewer errors per step than the SKP and neurosphere groups at 5 weeks and 9 weeks after injury, this difference did not reach significance (Fig. 2.12C).

Neuropathic pain after transplantation for spinal cord injury is of particular importance in light of a recent report suggesting that undifferentiated neural stem cells, but not predifferentiated progeny, resulted in an increased incidence of allodynia (Hofstetter et al.,
To ask whether SKP and SKP-SC transplants had any effect on the development of neuropathic pain, we first used the dynamic plantar aesthesiometer test to assess hindlimb withdrawal reflexes in response to mechanical stimulation (Fig. 2.13A). Interestingly, Kruskal-Wallis test revealed a significant effect of treatment at 5 weeks ($\chi^2_{(2)}=9.35$, $p<0.01$) and 7 weeks ($\chi^2_{(2)}=6.237$, $p<0.05$) after injury. At 5 weeks after injury, both neurosphere (26.2 ± 4.4 g) and SKP-transplanted animals (18.8 ± 2.8 g) showed a significant reduction in withdrawal thresholds compared with either their individual preinjury thresholds ($p<0.05$), or with the SKP-SC transplanted group (35.1 ± 3.2 g) ($p<0.05$). Although this hypersensitivity had resolved by 9 weeks this transient increase was potentially indicative of increased sensitivity to mechanical stimuli in the two groups transplanted with undifferentiated precursor cells. To pursue this further, we used the infrared heat test to assess sensitivity to temperature. Repeated measures ANOVA revealed significant effect of treatment ($F_{(2,37)}=2.38.06$, $p<0.001$) and an interaction of treatment by time ($F_{(2,70)}=2.33$, $p<0.04$). Interestingly, neither SKP-SC- or neurosphere-transplanted animals exhibited a change in withdrawal thresholds to heat at any point (Fig. 2.13B). However, animals treated with naïve SKPs showed significantly heightened sensitivity at both 7 weeks ($p<0.05$) and 9 weeks ($p<0.05$) relative to either their preinjury baseline, or to both SKP-SC- or neurosphere-treated animals ($p<0.05$). Thus, two independent measures demonstrated that transplanted SKP-SCs had no impact on sensitivity to sensory stimuli but that naïve SKPs produced heightened sensory responses (i.e., lowered threshold), potentially indicating the development of neuropathic pain.
Figure 2.13 SKP-derived SCs do not reduce sensory thresholds when transplanted into the contused spinal cord

Animals were assessed for hindlimb sensory thresholds prior to their injury, and 8 weeks after transplantation of neonatal SKPs, SKP-derived SCs, or forebrain SVZ neurospheres. (A) Results of the mechanical sensitivity test demonstrating that transplantation of SKP-derived SCs had no impact on mechanical pain thresholds. Interestingly, transplants of SKPs or neurospheres led to a transient reduction in mechanical thresholds that recovered to preinjury levels by 9 weeks after injury. (B) Results of the thermal sensitivity. Analysis of the data by repeated measures ANOVA revealed a significant week by treatment interaction (p < 0.03) and a main effect for treatment group (p < 0.01). Transplantation of SKP-derived SCs or neurospheres had no impact on sensitivity to heat, but transplantation of SKPs led to a significantly heightened sensitivity to heat at 7 weeks after injury that persisted at 9 weeks. In both panels, results represent mean ± SEM. *p<0.05.
2.4 Discussion

Here, we have performed a direct comparison of the neuroanatomical and functional outcomes after transplantation of SKPs, purified SKP-derived SCs, and CNS SVZ neurospheres into the contused rat spinal cord. This comparison provides strong evidence that SKP-SCs are a highly suitable transplant candidate for treatment of spinal cord injury. In particular, we demonstrate that transplanted SKPs and SKP-SCs display enhanced survival related to CNS neural stem cells, and fill or bridge the lesion cavity in the contused spinal cord. Transplanted naïve SKPs also responded to endogenous cues in the injured spinal cord and differentiate into SCs that myelinate spared and/or regenerating axons. However, despite this myelination, naïve SKPs do not appear to be good candidates for spinal cord repair, because they also differentiate into mesodermal cell types, cause long-term enhanced temperature sensitivity, and do not significantly enhance functional locomotor recovery relative to CNS stem cells. In contrast, transplantation of isolated, expanded SKP-SCs resulted in a more than threefold increase in myelination relative to naïve SKPs. These SKP-SC transplants maintained a rostral-caudal orientation, thereby providing an effective “bridge” across the lesion cavity, which supported robust axonal growth. SKP-SC transplants also promoted sparing of the tissue rim surrounding the lesion, and caused only minimal reactive gliosis. These positive neuroanatomical effects were associated with a significant improvement in locomotor function, with no alterations in mechanical or heat sensitivity. Thus, SKPs, predifferentiated into their SC progeny, represent a potentially autologous source of precursors for treatment of spinal cord injury.

Spinal cord contusion injury triggers a cascade of secondary events that lead to neuronal and oligodendroglial death and loss of tissue adjacent to the lesion over a period of hours to
weeks (Crowe et al., 1997; Hausmann, 2003; Jones et al., 2005; Schwab et al., 2006). In the
wake of this process a central cavity forms surrounded by an astrocytic scar, often leaving a
small rim of spared white matter with focal demyelination (Bunge et al., 1993). Therapies aimed
to treat this type of injury have focused on four different strategies; neuroprotection to reduce the
size of the lesion, promotion of long-distance axon regeneration and reconnection, neural
replacement/gray matter reconstitution, and remyelination of the spared axons (Enzmann et al.,
2006). In this regard, reconstituting conduction across demyelinated axons is currently
considered to be of particularly high priority (Blight, 2002) because substantial functional
restoration can be achieved using this approach (Felts and Smith, 1992; Waxman et al., 1994;
Guest et al., 1997a). Here, we focused on remyelination using SKPs, an accessible dermal source
of self-renewing neural crest-like precursors (Toma et al., 2001; Fernandes et al., 2004; Toma et
al., 2005) that can generate myelinating SCs (McKenzie et al., 2006). Although SCs are a PNS
glial cell, they myelinate CNS axons (Blakemore and Franklin, 1991; Gilmore and Sims, 1993),
provide a highly conducive environment for axon regeneration and enhance functional recovery
when transplanted into the injured spinal cord (Guest et al., 1997a; Xu et al., 1997; Keirstead et
al., 1999a; Xu et al., 1999; Pinzon et al., 2001; Pearse et al., 2004a; Pearse et al., 2004b). Our
findings indicate that SKP-SCs myelinate both spared and sprouting/regenerating axons in the
injured spinal cord, and promote functional locomotor recovery.

Data reported here indicate that both SKPs and SKP-SCs remyelinated the spinal cord,
but that significant functional improvement was only seen with SKP-SCs. This behavioural
difference is likely attributable to a combination of reparative or protective effects observed only
after treatment with SKP-SCs. First, the magnitude and location of remyelination differed
between these two groups; SKP-SCs provided increased myelination relative to SKPs, and this myelination occurred both within the transplant and in the spared tissue rim, whereas myelination by naïve SKPs was restricted to the transplant. Second, SKP-SCs provided significant preservation of tissue surrounding the lesion epicentre. Third, SKP-SCs modified the surrounding host environment, reducing reactive gliosis and decreasing neurocan expression. This was accompanied by migration of SKP-SCs into the surrounding host tissue, where they intermingled with host astrocytes at the lesion/host interface, apparently allowing a reciprocal movement of astrocytes into the transplant. Interestingly, this differs from transplants of mature nerve-derived SCs, where donor SCs and highly hypertrophied host astrocytes maintain discrete domains (Pearse et al., 2004a). This may occur because the SKP-SC population includes newly-developed SCs and perhaps even SC precursors. Recent work indicates that immature versus mature SCs have very distinctive properties (Jessen and Mirsky, 2002, 2005; Wanner et al., 2006) and these features may contribute to the enhanced axonal growth and reduced SC/astrocyte segregation observed here. Pacifying effects on the host astrocytes have also been observed after transplantation of olfactory ensheathing cells (Ramer et al., 2004c; Ramer et al., 2004b).

Previous studies have used other stem cells in an attempt to remyelinate the injured spinal cord, and several of these have shown concomitant recovery of locomotor function, including CNS neural stem cells (Ogawa et al., 2002; Vroemen et al., 2003; Cummings et al., 2005; Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006), glial restricted precursors (Cao et al., 2005) and embryonic stem (ES) cells differentiated to either a neural (Liu et al., 2000) or oligodendroglial fate (Keirstead et al., 2005; Nistor et al., 2005). These different sources all have
distinct advantages and disadvantages. CNS neural stem cells, when genetically transduced with neurogenin-2 (Hofstetter et al., 2005), administered coincidentally with growth factors (Karimi-Abdolrezaee et al., 2006), or transfected to express growth factors (Cao et al., 2005; Macias et al., 2006) demonstrated remyelination of the injured cord, and enhanced functional recovery. However, naive CNS neural stem cells, like the naive SKP transplants performed here, caused allodynia (Hofstetter et al., 2005). ES cells, including human ES cells, can efficiently generate oligodendrocytes and their precursors appear to remyelinate the injured cord and promote functional recovery (Keirstead et al., 2005), but one major issue associated with their use is that differentiated but unpurified ES cell transplants can cause tumor formation within the environment of the CNS (Roy et al., 2006).

The studies here indicate that SKP-SCs are a viable alternative that have a number of advantages. First, SKPs derive from the dermis, and thus represent an accessible, potentially autologous tissue source. In contrast, both ES cells and CNS neural stem cells are currently heterologous cell sources, and nerve-derived SCs must be harvested by invasive nerve biopsies. Second, SKPs represent an adult human precursor population, the use of which circumvents potential ethical issues. Third, human SKPs can be robustly expanded (Joannides et al., 2004; Toma et al., 2005), and SKP-SCs behave like immature, developing SCs, in contrast to nerve-derived SCs which are mature and thus have limited proliferation potential. However, despite these potential advantages, although human SKPs can be isolated from both neonatal (Toma et al., 2005) and adult (Joannides et al., 2004; Toma et al., 2005) skin, and although human SKPs make SCs (Toma et al., 2005; McKenzie et al., 2006), we have yet to develop protocols for successful isolation and expansion of SCs from human skin.
The studies reported here reinforce the growing consensus that naive stem cells are not the best alternative for nervous system therapies. Although transplanted naive SKPs differentiated into SCs, they also differentiated into mesodermal cell types, as they do in culture. SC differentiation likely occurred in response to cues deriving from demyelinated or newly-growing axons, something that we documented previously (McKenzie et al., 2006). The mesodermal differentiation likely also occurred in response to the many growth factors/cytokines that are present within the lesioned spinal cord (Nakamura and Bregman, 2001; Widenfalk et al., 2001; De Biase et al., 2005). A second undesirable outcome seen with transplanted naive SKPs was enhanced sensitivity to sensory stimuli, as was previously observed with CNS neural stem cells (Hofstetter et al., 2005). Thus, our findings, in concert with studies on neural stem cells (Hofstetter et al., 2005; Macias et al., 2006) and ES-derived cells (Roy et al., 2006), indicate that the best strategy for nervous system transplantation likely involves stem cell predifferentiation and subsequent purification of the desired cell type.

In summary, our results indicate that transplantation of SKP-SCs represent a viable alternative strategy for repairing the injured spinal cord, thus identifying a novel, accessible, and potentially autologous source of myelinating cells for nervous system repair.
Chapter 3:

Neonatal Schwann Cells Generated from Skin-derived Precursors or Peripheral Nerve Induce Similar Levels of Functional Recovery after Transplantation into the Partially Injured Cervical Spinal Cord of the Rat

2 A version of this chapter is in preparation for publication. Sparling JS, Bretzner F, Biernaskie J, Assinck P, Jiang Y, Arisato H, Plunet WT, Borisoff J, Liu J, Miller FD, Tetzlaff W. Neonatal Schwann cells generated from skin-derived precursors or peripheral nerve induce similar levels of functional recovery after transplantation into the partially injured cervical spinal cord of the rat.
3.1 Introduction

Cell transplantation is one of the more promising therapeutic approaches for reversing the loss of function that occurs following mammalian spinal cord injury (SCI) and Schwann cells harvested from peripheral nerve (N-SCs) have emerged as one of the leading transplant candidate cell types for potential clinical application (Tetzlaff et al., 2011). N-SCs stand out as a potential therapy for SCI because they are known to myelinate, as well as facilitate the growth of, CNS axons, and those cells have been shown to improve locomotor recovery in a variety of SCI animal models (reviewed in: Oudega and Xu, 2006; Fortun et al., 2009). In addition, those cells can be generated from autologous tissue sources in adult mammals, which obviates the need for potentially harmful immunosuppression (reviewed in Denton et al., 1999), and unlike some other autologous cellular therapies (e.g., induced pluripotent stem cells) (Czepiel et al., 2011), N-SCs carry very little risk of tumor formation (reviewed in Bunge and Wood, 2012).

There are however, drawbacks to the clinical application of autologous N-SCs, as those cells are typically harvested by excising 12-15 cm of sural nerve for transplantation in humans with SCI (Saberi et al., 2008; 2011), and that procedure causes a peripheral nerve injury that results in permanent sensory deficits in the foot/ankle region and carries the risk of painful neuroma formation (IJpma et al., 2006; Hood et al., 2009). Although those issues may go unnoticed in patients who lack lower limb sensation, not all humans with SCI have such deficits and if successful, N-SC therapy itself may result in a return of sensation for those who do.

Rather than sacrifice peripheral nerve in an effort to regain central function, we favour the use of an alternative source of autologous Schwann cells (SCs): skin-derived precursors (SKPs). SKPs are a multipotent precursor cell found in the dermis of rodent and human skin
(Toma et al., 2001; 2005). In rodents those cells have been shown to serve as a resident stem cell contributing to dermal maintenance, wound healing, and hair follicle morphogenesis in adult tissues (Biernaskie et al., 2009), and those cells can be differentiated into Schwann cells (SKP-SCs) \textit{in vitro} and expanded at high purity for the purposes of therapeutic transplantation (Biernaskie et al., 2006; McKenzie et al., 2006). Unlike nerve excision, the harvest of SKPs involves a simple skin biopsy, which carries no risk of permanent functional deficits or neuroma formation and could be performed during stabilization surgery after SCI; thereby avoiding an additional nerve excision surgery and providing a safe and accessible source of autologous SCs.

Previously we demonstrated that neonatal rodent SKP-SCs promote repair and functional recovery after delayed transplantation in rats with thoracic contusions (Chapter 2; Assinck et al., submitted). That work demonstrated that SKP-SCs share many of the behavioural properties and reparative effects displayed by adult N-SCs transplanted under similar conditions, but it also found evidence to indicate that SKP-SCs may have advantages over their nerve-derived counterparts in terms of their ability to integrate with astrocyte-rich spared host tissue and promote the growth of certain brainstem-spinal axons into and even through the lesion site.

Although SKP-SCs induced the growth of serotonergic and noradrenergic fibres, which are thought to arise primarily from the raphe nuclei and locus coeruleus of the brainstem, respectively, we noted that SKP-SCs alone were not able to induce a regenerative response from the corticospinal tract (CST) (Chapter 2; Assinck et al., submitted). In the present work we examined the influence of SKP-SCs on another major descending supraspinal motor system: the rubrospinal tract (RST); which is highly involved in skilled forelimb movements and overground locomotion in the rat (Muir and Whishaw, 2000). Towards that goal we used a left
cervical (C4/5) dorsolateral funiculus forceps crush model of SCI to examine the ability of SKP-SCs to promote sparing/plasticity of the RST. In order to establish the efficacy of SKP-SCs as a treatment for incomplete cervical SCI, we compared SKP-SC-transplanted rats to media-treated (Experiment 1) or fibroblast-transplanted (Experiment 2) control animals. In addition, we compared SKP-SCs to N-SCs side-by-side in the same SCI injury/transplantation experiment (Experiment 2) for the first time, in an effort to establish the suitability of SKP-SCs as a replacement for N-SCs in therapeutic applications. In light of our previous findings, we hypothesized that SKP-SCs would prove efficacious as a treatment for cervical SCI and we predicted that those cells would have advantages over N-SCs in terms of their reparative benefits and functional effects.

### 3.2 Materials and methods

All experimental procedures used in this work were approved by the Animal Care Committee of the University of British Columbia and/or the Hospital for Sick Children Research Institute in accordance with the guidelines of the Canadian Council on Animal Care. Two separate experiments were conducted; the first (Experiment 1) compared SKP-SC transplantation to media treatment alone and the second (Experiment 2) compared the effects of transplanting SKP-SCs, nerve-derived SCs (N-SCs) or dermal fibroblasts (Fibros). Although the same methods were used in both studies, these experiments were conducted over a year apart and involved different treatments and timelines (see Fig. 3.1A,B), so the results of each experiment are presented separately throughout this work.
3.2.1 Animals and experimental design

Transgenic Sprague Dawley (SD) rats expressing GFP (National Bio Resource Project Kyoto) served as the source of GFP-positive (GFP+) tissue used to generate all of the cells transplanted in this study. In light of the higher clinical prevalence of SCI in males (Wyndaele and Wyndaele, 2006), and because gender may influence spontaneous repair in adult rodents (Li et al., 2006), we chose to use only male rats as transplant recipients. A total of 97 adult male SD rats (300-500g) were used in this work, 10 of which were euthanized prior to endpoint due to surgical/health complications. Twenty-two wildtype SD rats (Charles River, St. Constant, QC) served as uninjured controls for behavioral, electrophysiological and/or axonal tracing analyses. To minimize animal use, both GFP-negative (GFP−) SD rats (UBC Animal Care Facility, Vancouver, BC) and wildtype (WT) SD rats (Charles River) were used in Experiment 1. Given that SD is an outbred rat strain and that the GFP− rats were all 6 or more generations removed from the animals used to generate the cells for transplantation in this study, all transplants in this study would be considered allogeneic. Thirty injured/treated rats reached the designated endpoint (media: n=10; SKP-SC: n=20) in Experiment 1. All of the animals used in Experiment 2 were WT SD rats (Charles River) and of the rats that were injured and treated in this study, 35 (SKP-SC: n=11; N-SC: n=13; Fibro: n=11) reached end-point. Half of the animals in each treatment group in Experiment 1 and all of the animals in Experiment 2 were immunosuppressed with Cyclosporine A (CsA; Novartis Pharmaceuticals, Mississauga, ON). CsA was administered via daily injection (10 mg/kg per day, i.p.) from 2 days before surgery until 2 weeks post-surgery, after which point oral CsA was provided in homecage drinking water (Neoral, Novartis; 1.5 ml/L of water) for the duration of the experiments.
3.2.2 Cell culture for transplantation

Three different cell types (SKP-SCs, N-SCs, and Fibros) were isolated / differentiated and expanded for transplantation into the injured dorsolateral funiculus (DLF) in this study. For ease of tracking transplanted cells and their progeny, all transplanted cells were generated from GFP⁺ neonatal SD rat tissue. The generation of SKPs and differentiation of SKP-SCs followed the same methods in both experiments. In the second experiment, pups that provided the skin to generate SKPs also provided the tissue to generate N-SCs and Fibros. All cells were passaged 3 to 4 times prior to transplantation.

3.2.2.1 Isolation of SKPs

SKPs were isolated and cultured as previously described (Biernaskie et al., 2006). Briefly, excised backskin from neonatal GFP-expressing rats was stripped of underlying muscle and adipose tissues, then minced and incubated in collagenase type XI (Sigma; St. Louis, MO) at 37°C for 1 hour. Digestion was inactivated by dilution in Dulbecco’s Modified Eagles Medium (DMEM; Invitrogen; Carlsbad, CA) and skin was mechanically dissociated to single cells and passed through a 40µm cell strainer to remove undigested epidermis. Skin cells were plated at an initial density of 50,000 cells/ml in proliferation medium consisting of DMEM-F12 (3:1; Invitrogen) medium supplemented with 2% B27 (Gibco), penicillin streptomycin (0.1%; Invitrogen), fungizone (40µg/ml; Gibco), bFGF (40 ng/ml; BD Biosciences; San Jose, CA) and epidermal growth factor (20 ng/ml; BD Biosciences).

3.2.2.2 Differentiation of SKP-SCs

Schwann cell differentiation was carried out as previous published (Biernaskie et al., 2006). Briefly, primary or secondary SKP spheres were digested with collagenase for 15 minutes at 37°C and triturated to single cells. Cells were then plated at 50,000 cells/ml on 10 cm plastic
tissue culture plates (BD Falcon; San Jose, CA) coated with laminin (20µg/ml) and poly-d-lysine (PDL; 200µg/ml; both BD Biosciences). SKPs were grown in proliferation medium for 3-5 days containing 10% fetal bovine serum (FBS; Calbiochem; Darmstadt, Germany), then the medium was switched to DMEM-F12 (3:1) with 2% N2 supplement (Gibco), 50 ng/ml neuregulin-1β (heregulin-β1; R&D Systems; Minneapolis, MN) and 5 µM forskolin (Sigma). Medium was changed every 4 days. Cultures generated with this protocol were greater than 95% positive for p75 (Biernaskie et al., 2006).

### 3.2.2.3 Isolation of nerve-derived Schwann cells

Nerve-derived Schwann cells (N-SCs) were isolated from neonatal sciatic nerve segments as mentioned above. Following dissection the nerve segments were minced and digested in collagenase type XI at 37°C for 30-60 minutes. Cells were liberated by gentle trituration, and the dissociated cells were plated on 10 cm dishes coated with laminin/PDL and grown in DMEM:F12 (3:1) containing 50 ng/mL neuregulin-1β, 4µg/ml insulin (Gibco), 5µM forskolin, 2% N2 supplement, and in some cases 1% FBS.

### 3.2.2.4 Isolation of dermal fibroblasts

Dermal fibroblasts (Fibros) were isolated from neonatal glabrous (i.e., non-hairy) skin off the ventral surface of the hindfeet. Epidermis was removed following a 15-minute incubation in trypsin-EDTA (0.25%; Invitrogen), and the isolated dermis was then digested in collagenase type XI for 30-60 minutes at 37°C. Cells were liberated by gentle mechanical dissociation grown adherently on 10cm dishes in DMEM:F12 (3:1) containing 5% FBS, 40 ng/ml bFGF and 1% B27 supplement.
3.2.3 Spinal cord injury and cell transplantation

3.2.3.1 Dorsolateral funiculus crush

Rats were anaesthetized with a mixture of ketamine hydrochloride (70 mg/kg, i.p.; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (10 mg/kg i.p.; Bayer Inc., Etobicoke, ON), a laminectomy was performed on the left side, exposing the fourth and fifth cervical segments. The dura was then cut with microscissors to expose the spinal cord on the left side. The DLF including the rubrospinal tract (RST) was crushed for 20 s with custom-designed fine surgical forceps at a depth of 1mm as described previously (Ramer et al., 2004c; Richter et al., 2005).

3.2.3.2 Cell transplantation

Immediately following injury, SKP-SCs (Expt 1 & 2), N-SCs (Expt 2), or Fibros (Expt 2) were transplanted into the spinal cord using procedures modified from our previous work (Bretzner et al., 2008; 2010). Briefly, adherent cells were detached from the dish by digestion in trypsin-EDTA (0.01%), then the trypsin was deactivated by adding 10% FBS and the cells were spun down and resuspended in fresh Hank’s Balanced Salt Solution (HBSS; Gibco; Carlsbad, CA; Expt 1) or DMEM (Expt 2) at approximately 135,000 cells/µl. Resuspended cells were drawn into a pulled glass pipette (with a diameter of 60-80µm) fitted to a 10 µl Hamilton microsyringe (Reno, NV). Three stereotaxic microinjections of 0.5µl of cell suspension were applied to the crush site, two at the medial edge (at 0.5 and 1 mm depths) and one at the lateral edge (0.5 mm depth) of the lesion for a total of 1.5µl containing 200,000 cells. Media control animals in Experiment 1 received equivalent injections of the same volume of HBSS without cells. The glass pipette was left in place for 5 minutes after each injection to ensure that cells
remained in the spinal cord and were not withdrawn with the syringe. After the final injection the muscle and skin were repositioned and sutured to close the wound. Rats received Ringer’s solution (5ml, s.c.) to maintain hydration and buprenorphine (0.3 mg/kg) to alleviate post-surgical pain. Rats that opened their wounds were also treated with antibiotics (Baytril, 0.06 ml, i.m.) to prevent infection.

### 3.2.4 Behavioural testing

In Experiment 1 behavioral testing using the cylinder test was used to assess recovery of forelimb locomotor function at 6 and 10 weeks post injury (wpi; Fig. 3.1A). Logistical complications prevented baseline testing of the animals prior to injury, so an uninjured control group was assessed to provide a proxy for baseline (i.e., pre-injury) behavior. In Experiment 2, cylinder and CatWalk (Noldus, Netherlands) data were collected prior to injury and again at 6, 8, and 10 wpi (Fig. 3.1B), and pre-injury data collection eliminated the need for an uninjured behavioral control group. In both experiments, electrophysiological measures and axon tracing procedures were conducted within days of the 10 wpi behavioral assessments and precluded additional behavioral testing. All behavioral data were scored by raters blinded to the experimental treatment given to each animal.

#### 3.2.4.1 Cylinder test

The use of each forelimb during vertical exploration was assessed by videotaping animals’ rearing behavior in a closed Plexiglas cylinder (20 cm in diameter X 30 cm high) (Liu et al., 1999). Forelimb use was scored by counting the number of times an animal contacted the wall of the cylinder using the left forelimb only (affected by injury), the right forelimb only (unaffected by injury), or both forelimbs simultaneously during rearing. To reflect the overall use
of the forelimb affected by injury more accurately, the final data for each animal was expressed as the percentage of use of the “left plus both” forelimbs relative to the total number of forelimb contacts.

### 3.2.4.2 CatWalk

As a further measure of locomotor control, we used the CatWalk device to analyze a wide variety of forelimb and hindlimb gait parameters (Hamers et al., 2001; 2006). This device is capable of capturing the shape, size, orientation and timing of each paw placement, as well as providing a measure of the pressure each paw exerts on the glass (i.e., paw intensity). The animals were trained to cross the walkway in a darkened room and we recorded multiple crossings for each animal at each timepoint for subsequent analysis using CatWalk v7.1 software. We averaged values from five steady runs with a minimum of three complete step cycles per animal at every timepoint, and those values were used to generate group averages for subsequent statistical analysis.

### 3.2.5 Electrophysiology

Immediately following 10 wpi behavioral testing, the synaptic efficacy of the RST was assessed by recording electromyographic (EMG) responses in the left forelimb (affected by injury) evoked by electrical stimulation of the red nucleus. Rats were anaesthetized with a mixture of ketamine hydrochloride (70 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg i.p.), and placed in a stereotaxic frame. Additional doses of ketamine were given if the rat responded to a palpebral stimulus or a hindpaw pinch. EMG responses were recorded via bipolar electrodes made of insulated copper microwires with exposed tips of 2 to 3 mm inserted into the left forearm (extensor digitorum communis or extensor carpi radialis) muscles. A tungsten electrode
was then lowered into the red nucleus at a depth of 7mm below the dura, through an opening in the skull at 6.1mm posterior to Bregma and 1.7mm lateral (right) of the midline. The optimal stimulation site was located by varying the depth of the electrode until EMG responses were elicited by test stimulations. This was the location at which EMG responses in the forelimb muscles were evoked using the lowest current strength possible (i.e. motor threshold). Animals that failed to provide EMG responses at ventral stimulation coordinates within 1mm of the average depth of the red nucleus (from surface) were excluded from this analysis. A short train of eleven cathodal stimuli was applied at a frequency of 330Hz, with a pulse-duration of 0.2ms to evoke motor and EMG responses. EMGs were recorded using a Pentusa neurophysiology workstation (Tucker-Davis Technologies, Alachua, FL). EMGs were digitized at a sampling rate of 5 kHz and bandpass filtered between 5 and 500 Hz to eliminate background noise. The latency of evoked EMG responses was measured with respect to the onset of stimulation at the motor threshold and averaged from at least 15 EMG traces per animal.

3.2.6 Anterograde tracing of rubrospinal axons

Immediately following the completion of the electrophysiological measures, anterograde axonal tracing was conducted by stereotaxically injecting biotinylated dextran amine (BDA; 0.6µl, 10 kDa, 25% in 0.5% dimethylsulfoxide, Molecular Probes, Eugene, OR) into the red nucleus on the right side of the brain at a rate of 80nl/min via a pulled glass pipette (diameter of 20µm) fitted to a Hamilton microsyringe. The coordinates used for these injections were the same as those used for red nucleus stimulation (6.1mm posterior to Bregma, 1.7mm lateral (right) of the midline) but depth of the injection was kept constant at 7mm below the dura. The pipette remained in place for 5 minutes after injection to ensure that BDA was not withdrawn.
with the syringe. Six uninjured control animals were also subjected to BDA tracing, concurrent with the tracing of animals in Experiment 2, in order to establish baseline levels for RST axon density measures.

### 3.2.7 Tissue processing and immunohistochemistry

Eleven weeks after injury and treatment (one week after electrophysiological measures and BDA tracing), rats were anesthetized with a lethal dose of chloral hydrate (100 mg/kg, i.p.; BDH Chemicals, Toronto, ON) and perfused transcardially with phosphate buffered saline (PBS) followed by phosphate buffered, 4% paraformaldehyde (pH 7.4). Cervical segments of spinal cord were dissected, post-fixed in 4% paraformaldehyde overnight, cryoprotected in 24% sucrose in 0.1M phosphate buffer over 2-3 days, and frozen in isopentane over dry ice. Spinal cords were cut into 20μm sections on a cryostat, mounted on Superfrost Plus slides (Fisher, Houston, TX) and stored at -80°C. Cervical segments C2 and C7 were cut into 20μm sections in the coronal plane. Cervical segments from C3 to C6 were cut into 20μm longitudinal sections in the horizontal plane. For immunohistochemistry, frozen sections were thawed at room temperature for 30-60 minutes, rehydrated in 0.01M PBS for at least 10 minutes, then incubated for with 10% normal donkey serum and/or 10% normal goat serum containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 30 minutes to block nonspecific binding. Sections being immunolabelled for myelin protein zero (P₀) underwent alcohol delipidation to increase epitope availability prior to serum block. Primary antibodies were diluted in 0.01M PBS containing 0.1% Triton X-100 and incubated on tissue overnight at room temperature or 4°C. Following primary antibody incubation the tissue was washed and secondary antibodies (diluted in 0.01M PBS alone) were applied for 2-3 hours at room temperature, followed by multiple washes and cover-
slip application. The following primary antibodies were used: chicken anti-GFP (1:1000, Chemicon, Temecula, CA), rabbit anti-GFP (1:1000, Chemicon), goat anti-GFP (1:400, Rockland Immunochemicals, Gilbertsville, PA), mouse anti-glial fibrillary acidic protein (GFAP; 1:400, Sigma), rabbit anti-GFAP (1:1000, Dako, Carpentaria, CA), goat anti-GFAP (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-neurocan (1:100, Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-neurofilament 200 (NF; 1:500, Sigma), mouse anti-βIII-tubulin (βIIItub; 1:400, Sigma), rabbit anti-serotonin transporter (SERT; 1:500, Sigma), sheep anti-tyrosine hydroxylase (TH; 1:200, Chemicon), chicken anti-protein zero (P₀; 1:100, Aves Labs, Tigard, OR), mouse anti-rat p75 neurotrophin receptor (p75; 1:500, Chemicon), rabbit anti-p75 (1:400, Chemicon), rabbit anti-laminin (1:200, Sigma), mouse anti-fibronectin (1:200, Dako), and rabbit anti-collagen type I (Collagen; 1:60, Chemicon). Secondary antibodies included: FITC-, Cy3-, and AMCA-conjugated donkey anti-chicken, mouse, or rabbit antibodies (all 1:200, Invitrogen); Alexa 488-conjugated goat anti-chicken (1:200, Invitrogen); Donkey anti-chicken, mouse, rabbit, goat, or sheep antibodies conjugated to Dylight 405 (1:200), Dylight 488 (1:400), Dylight 594 (1:200), or Dylight 649 (1:300) (all Jackson ImmunoResearch, West Grove, PA). BDA was visualized by using Cy3- or AMCA-conjugated streptavidin (1:400, Jackson ImmunoResearch) applied for the duration of secondary antibody incubation. In some sections we also stained nuclei with Hoechst (Ho; 1:5000, Invitrogen), which was applied for 5 minutes following secondary antibody incubation.
3.2.8  Image analysis and histological quantifications

3.2.8.1  Image acquisition and processing

Immunofluorescence was digitally captured using an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada) or a Zeiss AxioObserver Z1 confocal microscope fitted with a CSU-X1 spinning disc (Yokogawa Electric, Tokyo, Japan) and AxioVision 4.8 (Zeiss). Digital images were processed for presentation and quantification using Photoshop 7.0 or 9.0 (Adobe Systems, San Jose, CA), SigmaScan Pro 5 (SPSS Inc., Chicago, IL), or Matlab 6.5 (The MathWorks, Inc., Natick, MA) software. Rostral is up and caudal is down in all of the sample images in every figure in this work, except Figure 3.4N and O, where rostral is right and caudal is left. All histological analyses were conducted by individuals blinded to the treatment of each animal.

3.2.8.2  Area and volume quantifications

Lesion, cavity, and GFP\(^+\) graft areas were quantified every 320μm through the extent of injury site by outlining the GFAP-negative (GFAP\(^-\)) area, the area devoid of cytoarchitecture, or the GFP\(^+\) area within the spinal cord, respectively. The total number of pixels within each of those areas was measured using SigmaScan Pro5 and the total lesion, cavity, and GFP\(^+\) graft volume were estimated for each animal using the following formula: Volume = Σ(area X section thickness X number of sections between samples). Using similar methods we estimated the GFAP\(^+\)/GFP\(^+\) volume for each animal and used that to calculate the percentage of the lesion occupied by the GFP\(^+\) graft for each animal. For a subset (n=6) of random animals from each group we also used similar methods to those described above to measure the P\(_0\)-positive (P\(_0\)^+\)) and GFP\(^+\) areas, as well as the area of overlap (both P\(_0\)^+\) and GFP\(^+\)). Importantly, we thresholded
images to eliminate any background signal to ensure that we were only measuring pixels associated with GFP\(^+\) and P\(_0^+\) structures prior to assessing overlap, and we were careful to ignore artifacts that did not have appropriate morphology. Those area values were used to calculate the percentage of GFP\(^+\) area that was also P\(_0^+\) (an estimate of the production of SC myelin by transplanted cells and their progeny) and the percentage of P\(_0^+\) area that was GFP\(^-\) (an estimate of how much of the SC myelin came from endogenous SCs).

### 3.2.8.3 Immunoreactivity quantifications

GFAP- and neurocan- immunoreactivity (IR) were evaluated by using SigmaScan Pro5 to measure the average fluorescence intensity within a 50\(\mu\)m wide line circumscribing the entire rostral or caudal edge of the lesion site on 3 longitudinal sections spaced 320\(\mu\)m apart in each animal. Image settings were chosen to avoid oversaturation of pixels adjacent to the lesion and care was taken to avoid artifacts and the very edge of the spinal cord. The measurement line drawn on a given section was also used to measure the intensity of an analogous area on the uninjured side of the spinal cord and those control values were used to normalize the intensity measurements in each section to account for potential differences in immunoreactive labelling of tissue sections from animal to animal. Normalized values from each section were averaged to attain mean GFAP-IR and neurocan-IR scores rostral and caudal to injury for each animal. To ensure that the intensity of neurocan labelling in the gray matter (where neurocan expression is much higher) was not masking group differences in the white matter we repeated the neurocan analysis measuring intensity in white matter regions only. There were no significant differences in neurocan-IR using either method, so here we present the neurocan-IR measured in the white matter only.
3.2.8.4 Gray and white matter RST axon quantifications

The level at which the RST branches in the gray matter was located by examining longitudinal tissue sections (every 20 µm in 180 µm blocks spaced 140 µm apart) between the dorsolateral funiculus and the central canal to find the highest concentration of BDA-positive (BDA⁺) RST axons branching in each animal. We measured axon density in the gray matter at that level in 3 longitudinal sections spaced 60 µm apart and in 3 coronal sections spaced 60 µm apart at C2. BDA⁺ RST axons within the gray matter 1mm rostral (‘far rostral’), immediately rostral (‘rostral’), and immediately caudal (‘caudal’) to the injury site at C4/5 were imaged from each longitudinal section, and the left DLF and adjacent gray matter were imaged on each coronal section. The RST axon density measurement used here was adapted from Ramer et al. (2004a). In brief, threshold overlays were created for each of those images, in which all pixels associated with BDA⁺ axons were assigned a grayscale value of 68 and all other pixels were assigned a value of 0, and gray matter RST axon density (‘RST density’) values were generated by dividing the average intensity of a given region of each overlay by 68. An average of 3 measures at each level provided the final raw RST density values in the gray matter at C2, far rostral, rostral and caudal levels of analysis for each animal. In addition, we counted BDA⁺ RST axon profiles in the white matter at C2 (‘axon counts’) to measure of the number of descending RST axons in the DLF that successfully transported BDA. Animals with axon counts <100 were excluded from analysis due to catastrophic failure of BDA tracing and animals with damage to the DLF in C2 coronal sections were excluded from the axon count analysis.

As a preliminary analysis of the RST data, we compared the BDA⁺ axon counts in the white matter at C2 between the two experiments and found that the animals traced in Experiment
1 had significantly more BDA\(^+\) axons than those traced in Experiment 2 \((t_{52})=-4.24, p=0.000;\) data not shown). This result is best explained by systematic differences in the efficacy of BDA tracing between the two experiments, as the animals were traced >1 year apart. In support of that notion, it was found that even the two groups that received the same treatment in both experiments (i.e., SKP-SC transplantation groups) differed significantly \((U_{20}=16.00, p=0.010)\) on that measure (data not shown). Further analyses revealed that there were no significant differences in the axon counts among the groups in Experiment 1 (media or SKP-SC) regardless of CsA administration (see Results and Fig. 3.6C), so the RST density measures were normalized to (i.e., divided by) the axon counts for each animal in Experiment 1 to eliminate any spurious variability due to animal-to-animal differences in BDA tracing efficacy from that data. The same cannot be said of the results in Experiment 2, where we found significant differences among the various groups on RST axon counts in the white matter (see below and Fig. 3.6H), which prevented us from normalizing the raw RST density data in the same manner, as doing so would eliminate not only potential differences in BDA tracing efficacy, but also apparent group differences in BDA labelling that may reflect different degrees of RST atrophy among the groups (see Results and Fig. 3.6H).

In light of the systematic difference in BDA labelling between the two experiments, and in the absence of an effective method of normalizing the data in Experiment 2, it is inappropriate to compare the groups from Experiment 1 (media or SKP-SC) to those from Experiment 2 (SKP-SC, N-SC, or Fibros) on any of the RST density variables included in this study. Given that all of the uninjured control animals were traced alongside the animals in Experiment 2, this finding also precludes comparisons between Experiment 1 treatment groups and the uninjured control
group. As such, we present the RST density results separately for each experiment, and we use normalized RST density values for Experiment 1 (Fig. 3.6D-G) and raw RST density values for Experiment 2, including the uninjured control group (Fig. 3.6H-L).

3.2.8.5 Spared rim width

The width of the spared tissue rim was assessed by measuring the thinnest width of the intact/continuous GFAP+ tissue separating the outside edge of the spinal cord from the inside edge of the lesion on two longitudinal GFAP-labelled sections 320μm apart near the lesion epicentre (defined as the level of maximal GFAP-negative lesion area). This was done using the distance measure on SigmaScan Pro5, and the two thinnest spared rim distances from those sections were averaged to provide a measure of the amount of intact tissue available as a substrate for axonal growth.

3.2.9 Statistical analysis and data presentation

All statistical analyses presented herein were conducted using SPSS 19/20 (IBM; Armonk, NY) and all graphical representations of data were made using GraphPad Prism 5 (GraphPad Software Inc., CA) and processed using Photoshop CS2 (Adobe). All behavioral data are presented as mean ± SEM and analyzed using independent-samples t-tests (Expt. 1) or repeated-measures analysis of variance (RM-ANOVA; Expt. 2), as appropriate. Significant RM-ANOVA effects were followed up with 1-Way ANOVAs and conservative post hoc testing (Bonferroni) at individual timepoints as appropriate. All of the non-behavioral data sets in this study had at least one group that violated the assumptions of normality and/or homogeneity of variance that must be met to conduct parametric analyses, so all electrophysiological and histological analyses were conducted using non-parametric tests and are presented as individual
data points with group median values indicated by black lines on the graphs. For independent tests involving 2 or more groups (i.e., comparisons among the treatment groups in Experiment 2), we used the Kruskal-Wallis ranked sum test ($\chi^2$), followed by pairwise testing with the Mann-Whitney $U$ test ($U$). The latter test was also used to compare each injured/treated group in both experiments to the uninjured control group where appropriate. Correlational analyses were conducted using parametric (Pearson’s correlation coefficient [$r$]) or nonparametric (Spearman’s rank correlation coefficient [$\rho$]) tests as appropriate for the particular variables in question and the groups included in said analysis. Given the conservative nature of non-parametric tests, alpha level corrections were not applied to any non-parametric analyses. Where appropriate, the degrees of freedom for statistical tests are provided in brackets. The significance level for all tests was set at $p < 0.05$ and 2-tailed test results were reported for all analyses.

3.3 Results

Here we report the results of two experiments, the first assessing the efficacy of SKP-SCs as a treatment for incomplete cervical SCI and the second comparing SKP-SCs to SCs generated from peripheral nerve (N-SCs) or dermal fibroblasts (Fibros) in the same injury model. As depicted in Figure 3.1, both experiments used an acute transplantation paradigm and included behavioral testing out to 10 wpi, followed by electrophysiological measures and rubrospinal tract (RST) axon tracing prior to endpoint at 11 wpi. Experiment 1 (Fig. 3.1A) compared acute SKP-SC transplantation to media treated controls and included behavioral testing conducted at 6 and 10 wpi, whereas Experiment 2 (Fig. 3.1B) involved the transplantation of SKP-SCs, N-SCs, or Fibros, and included additional behavioral assessments at pre-injury, and 1 and 8 wpi. Note that
Figure 3.1 Experimental Design

A: Experiment 1 timeline. Behavioral testing was conducted at 6 and 10 weeks after left C4/5 DLF crush injury and immediate treatment with SKP-SCs or media. The synaptic efficacy of the RST was assessed 1-2 days after week 10 behavioral testing and was followed immediately by BDA tracing of the RST. All rats were perfused for subsequent histological analyses one week after tracing. B: Experiment 2 timeline. The second experiment involved transplantations of SKP-SCs, N-SCs, or fibroblasts, and followed the same timeline as Experiment 1, but with the addition of behavioral testing prior to injury and at 8 weeks post-injury.
half of the animals in each group in Experiment 1 and all of the animals in Experiment 2 were given immunosuppression with Cyclosporine A (CsA; Fig. 3.1A,B). Regardless of treatment with SKP-SC or media in Experiment 1, there were no significant differences between groups of rats that received CsA and those that did not on any of the behavioral, electrophysiological, or histological outcomes, except the RST axon density analysis (see details below). As such we pooled data from CsA and no CsA groups to compare all the animals that received SKP-SCs to all media treated animals in every analysis from Experiment 1, except the RST measure, where CsA treatment was included as a variable in the analysis.

3.3.1 SC transplantation improves functional recovery after cervical SCI

3.3.1.1 Cylinder test results

We investigated total use of the forelimb affected by injury, by analyzing the percent use of “left & both” forelimbs during vertical exploration (i.e., rearing behavior) in the cylinder test in both experiments (Fig. 3.2A,B). Consistent with previous studies (Schallert et al., 2000; Bretzner et al., 2008; 2010), we found that uninjured control animals used their left forelimb or both forelimbs on 70-75% of contacts (dashed lines in Fig. 3.2A), as did the animals in Experiment 2 prior to injury (Fig. 3.2B – Pre-injury). At 6 wpi, the percent use of left & both decreased to a mean of ~55% in Experiment 1 regardless of treatment, but at 10 wpi the SKP-SC group used the left forelimb at significantly greater frequency than the media group ($t_{(27)}=-2.27$, $p=0.031$; Fig. 3.2A). In Experiment 2 total use of the left forelimb significantly declined after injury in all groups (see 1 wpi data Fig. 3.2B) and remained significantly lower than pre-injury values across all subsequent timepoints tested (RM-ANOVA within-subjects effect, $F_{(4,112)}=10.40$, $p=0.000$) regardless of treatment. Although both SC groups had higher average
Figure 3.2  Schwann cell transplants improve functional recovery following incomplete cervical spinal cord injury

A: Cylinder test results from Experiment 1. At 10 wpi, SKP-SC treated animals used the forelimb affected by injury (left) significantly more than media controls. Dashed lines depict the mean and SEM of uninjured control animals. B: Cylinder test results from Experiment 2. There were no significant differences in total left forelimb usage among the groups at any of the timepoints assessed. C: CatWalk results from Experiment 2. Mean forelimb intensity difference (left forelimb minus right forelimb; LF-RF) measured in arbitrary units (a.u.) was significantly less negative for the SKP-SC and N-SC groups compared to the Fibro group at 6 and 10 wpi, indicating that transplantation of SCs from either source promoted better weight support on the left forelimb (relative to the right) than fibroblast transplantation. The dashed line indicates a difference score of zero – the theoretical norm for intensity differences. A-C: *p < 0.05 and all results depicted as group means ± SEM.
total use of the left forelimb than the Fibro group at every time timepoint, there were no significant differences among the treatment groups. These results indicate that SKP-SC transplantation significantly enhanced use of the forelimb affected by injury when compared to media treatment (Experiment 1), but not in comparison to N-SC or Fibro treatment (Experiment 2).

### 3.3.1.2 CatWalk analysis results

We analyzed a variety of typical gait parameters (e.g., base of support, stride length, paw angle, and regularity index) from the CatWalk data collected in Experiment 2, but only found treatment-related effects on forelimb intensity difference scores (left forepaw intensity minus right forepaw intensity). Intensity is a measure of pressure applied by each paw (Vrinten and Hamers, 2003), so this can be interpreted as a difference in weight support provided by the forelimb affected by injury (left) and the forelimb not affected by injury (right). Forelimb intensity difference displayed a significant negative shift in all values after injury, indicating that less weight was applied to the forelimb affected by injury (left) than the forelimb not affected by injury (right). Although this difference in relative weight support was maintained for the duration of the experiment in all animals regardless of treatment (RM-ANOVA within-subjects effect, $F_{(4,124)}=37.14, p=0.000$), there were significant differences among the treatment groups over time as well (RM-ANOVA interaction, $F_{(8,124)}=2.30, p=0.025$). Follow-up ANOVAs revealed significant group differences at 6 and 10 wpi ($F_{(2,31)}=4.378, p=0.021$ and $F_{(2,31)}=6.936, p=0.003$, respectively), and Bonferroni post hoc tests revealed that both SKP-SC and N-SC groups had significantly smaller forelimb intensity difference scores than the Fibro group at 6 wpi ($p=0.044$ and $p=0.041$, respectively) and 10 wpi ($p=0.047$ and $p=0.003$, respectively). Importantly, the
SKP-SC and N-SC groups did not differ significantly at any timepoint. These results indicate that rats treated with either type of SC show better functional outcomes than rats treated with fibroblasts, as the SKP-SC and N-SC transplanted animals apply their body weight more evenly to both forelimbs. Furthermore, the lack of significant differences between the SKP-SC and N-SC treated groups indicates that SCs from either source provide similar benefits after incomplete cervical SCI.

3.3.2 SC transplantation improves rubrospinal efficacy after cervical SCI

Given that the RST plays a specific, preferential role in the control of distal forelimb muscles (Küchler et al., 2002), we chose to complement our behavioral assays of forelimb function by assessing RST efficacy using EMG recordings of forearm muscles (extensor digitorum communis or extensor carpi radialis) during stimulation of the red nucleus (the origin of the RST). EMG responses within distal muscles of the left forelimb were evoked by stimulation of the red nucleus in uninjured controls and at 10 wpi in injured/treated animals (see Fig. 3.3A for sample trace).

3.3.2.1 Motor threshold results

In Experiment 1, the media treated group had significantly higher distal forelimb motor thresholds than uninjured controls \((U_{(22)}=11.00, p=0.003)\), whereas the SKP-SC treated animals did not differ from either of those groups (Fig. 3.3B - Expt1 data). Experiment 2 demonstrated similar results, with only the Fibro group demonstrating significantly higher motor thresholds than the uninjured group \((U_{(22)}=32.00, p=0.048)\), and no significant differences between SKP-SC and N-SC groups or between either SC group and the uninjured controls (Fig. 3.3B – Expt2).
Figure 3.3 Schwann cell transplants increase rubrospinal efficacy after spinal cord injury

A: Sample EMG trace based on the average of 15 responses recorded in the distal musculature (extensor carpi radialis) of the left forelimb and evoked by stimulation of the contralateral red nucleus. The stimulus artifact has been removed for clarity. B: Motor threshold (i.e. minimum current strength; µA) required to evoke EMG responses in distal forelimb muscles. Only the media treated group (Expt 1) and the Fibro treated group (Expt 2) differed significantly from uninjured controls at 10 wpi. C: Latency (in milliseconds) of EMG responses evoked at motor threshold stimulation levels. All groups in both experiments had significantly longer EMG latencies than the uninjured control group, except for the SKP-SC treated groups, which had near-uninjured latencies in both experiments. B-C: * p < 0.05, ** p < 0.01, *** p < 0.005 and all results depicted as individual data points with group medians indicated by solid black lines (–).
3.3.2.2 EMG latency results

The results of Experiment 1 revealed that the latencies of evoked EMG responses in the distal muscles were significantly longer in the media group compared to the uninjured group ($U_{19}=12.00, p=0.009$), whereas the SKP-SC group did not differ from either of the other groups (Fig. 3.3C – Expt1). Experiment 2 revealed that both the N-SC and Fibro groups had significantly longer EMG latencies than the uninjured control group ($U_{24}=36.50, p=0.016$ and $U_{21}=28.00, p=0.038$, respectively), whereas the SKP-SC group did not differ significantly from uninjured controls (Fig. 3.3C – Expt 2) and there were no significant differences among the three treated groups.

In summary, following DLF crush, the efficacy of the RST is diminished as stronger stimulation of the red nucleus is required to evoke EMGs in the distal forelimb muscles and those EMG responses take longer to reach target muscles. Treatment with media or dermal fibroblasts failed to improve either measure of RST efficacy, whereas the transplantation of either type of SC returned motor thresholds to levels that did not differ significantly from uninjured controls. In addition, SKP-SC transplantation alone improved the speed of conduction, as EMG latencies for SKP-SC treated animals in either experiment did not differ significantly from uninjured control values.

3.3.3 Cavitation persists after acute transplantation of SCs, but not fibroblasts, in the partially injured cervical spinal cord

To characterize the neuroanatomical effects of acute treatment after unilateral DLF crush injury, we examined lesion and cavity volume as indicators of pathology at 11 wpi. In media treated animals, the lesion was characterized by a substantial cavity starting in the gray matter at
the level of the DLF and extending to the edge of the spinal cord through the area normally occupied by the RST (Fig. 3.4A - Media). A small rim of spared tissue was usually left separating the cavity from the outside of the spinal cord and tissue bridges were occasionally found spanning the ventral portion of the cavity. SKP-SC transplanted animals showed a similar injury morphology, and did not differ significantly from media treated animals with respect to lesion or cavity volume (Fig. 3.4A, and 4B,C – Expt 1); although it was noted that SKP-SC transplanted animals (in either experiment) had more/larger mid-lesion tissue bridges spanning the lesion site (see Fig. 3.4A, 4F′, and 4L-L′′), similar to the N-SC group in Experiment 2 (see Fig. 3.4A, 4F′′, and 4M-M′′).

With the exception of cavity volume in the Fibro group, the lesion and cavity volumes generally appeared similar between Experiment 1 and 2 (compare Expt1 and Expt2 data in Fig. 3.4B,C), which suggests that injury severity was roughly equivalent in the two studies. Lesion volume did not differ significantly among the groups in Experiment 2 (Fig. 3.4B; Expt 2), but cavity volume did ($\chi^2(2)=19.44$, $p=0.000$), as the Fibro group showed significantly less cavitation than the SKP-SC or N-SC groups ($U_{(17)}=0.00, p=0.000$ and $U_{(19)}=0.00, p=0.000$, respectively; see Fig. 3.4C – Expt 2). Similar results were found when only animals that showed survival of transplant-derived cells were assessed, as the percent of lesion occupied by cavity differed significantly among the groups in Experiment 2 ($\chi^2(2)=20.51$, $p=0.000$), with the Fibro group showing significantly less cavitation than either the SKP-SC or N-SC groups ($U_{(17)}=0.00, p=0.000$ and $U_{(19)}=0.00, p=0.000$, respectively; Fig. 3.4E – Expt 2). Indeed, most of the animals (7/9) in the Fibro group showed no cavitation whatsoever, as their lesion sites were filled with cells (indicated by nuclear staining in Fig. 3.4H), extracellular matrix (ECM) molecules such as
Figure 3.4 Cellular responses and lesion properties 11 weeks after injury/transplantation

A: Representative images (based on median graft/lesion data) of the lesion epicentre in GFAP- (magenta) and GFP- (green) immunolabelled tissue; arrows and arrowheads denote GFP⁺ SCs in the spared rim and fragments of mid-lesion bridges, respectively. B-D: Lesion (B), cavity (C), and graft (D) volume (mm³) data (group medians depicted as solid black lines [–]). E: Percent of lesion (white) occupied by cavity (black) and graft (green) (depicted as means ± SEM) for tissue containing GFP⁺ cells at endpoint. The Fibro group showed significantly reduced cavitation (C) and graft survival (D) compared to either SC group (Expt 2), but when fibroblasts survived transplantation they occupied an average percent of lesion volume similar to that occupied by transplanted SCs (E). F-F′′: Sample images of GFAP- (magenta) and GFP- (green) immunolabelled tissue depicting higher than median graft survival in Experiment 2 groups; SCs preferentially occupied tissue in the spared rim (arrows) and mid-lesion bridges (arrowheads). G-K: Sample images of Fibro transplanted tissue. Regardless of graft survival, fibroblast transplantation resulted in filling of the lesion cavity by endogenous cells (indicated by Hoechst nuclear staining [H] and including P₀⁺ SCs [G]) and ECM molecules (including fibronectin [I], collagen type-I [J], and laminin [red; K]). L-Q: Fluorescent (L-M′′) and/or confocal (N-Q) micrographs of GFP- (green) and P₀- (red) immunolabelled tissue from SKP-SC (L-L′′, N, P) and N-SC (M-M′′, O, Q) treated animals, with or without NF/βIIIltub- (blue; axons; N,O) or p75- (blue; P,Q) immunolabelling. Endogenous (P₀⁺/GFP⁺) SCs were occasionally found in both groups (e.g., arrows in L-L′′ and N), but the majority of P₀⁺ SC myelin was found in areas occupied by transplant-derived cells (arrowheads in L-Q). Transplanted SKP-SCs and N-SCs both generated non-myelinating (p75⁺/P₀⁺; * in N, O; arrows in O-Q) and myelinating (P₀⁺/p75⁺; arrowheads in N-Q) SCs in vivo. B-E: * p < 0.05, *** p < 0.005.
fibronectin (Fig. 3.4I), collagen type I (Fig. 3.4J), laminin (Fig. 3.4K), and neurocan (Fig. 3.5B – Fibro), as well as endogenous SCs (labelled with P₀ in Fig. 3.4G). The lack of cavitation and the presence of cells and ECM in the lesion site occurred in all fibroblast transplanted animals regardless of graft survival and presumably resulted from the invasion of the lesion site by fibroblasts and other cells that normally reside in the meninges. Similar results have been observed previously following the acute transplantation of skin fibroblasts into dorsal column transections at the cervical level (Vroemen et al., 2007).

3.3.4 Transplanted neonatal SCs survive and primarily occupy the spared tissue rim and mid-lesion bridges in the crushed DLF

Similar to our previous work (Chapter 2), nearly all of the animals (15/16 in Expt1 and 10/10 in Expt2) that received SKP-SC transplants showed some degree of graft survival (Fig. 3.4D). However, that survival varied considerably for both SC treated groups (compare Fig. 3.4A to Fig. 3.4F′,F″) and appeared to be diminished compared to that seen after delayed transplantation into thoracic contusions (Chapter 2), as we transplanted 1/5 the number of SKP-SCs in the present experiments, but only saw 1/10 the graft volume at endpoint. T-cell-dependent immune rejection appeared to play a minimal role in cell loss, as graft volume was not significantly higher in SKP-SC transplanted animals that received CsA compared to those that did not in Experiment 1 (data not shown). The lack of improved cell survival in the presence of CsA was confirmed in Experiment 2, as graft volume did not differ significantly between the SKP-SC groups in Experiment 1 and Experiment 2 (Fig. 3.4D). Graft volume also did not differ significantly between the SKP-SC and N-SC groups in Experiment 2 (Fig. 3.4D); indicating
similar levels of survival of transplant-derived cells in all SC treated groups in the present experiments, regardless of the source of the cells.

Graft volume was significantly lower in the Fibro group compared to both SKP-SC and N-SC groups ($U_{(17)}=20.00$, $p=0.037$ and $U_{(19)}=26.50$, $p=0.046$, respectively; Fig. 3.4D – Expt 2), as many fibroblast transplanted animals (6/9) had no GFP$^+$ cells within the spinal cord at 11 wpi. When transplanted fibroblasts did survive, they occupied a percent of the lesion volume that did not differ significantly from that occupied by transplanted SCs (Fig. 3.4E – Expt 2). Within the spinal cord, the vast majority of GFP$^+$ fibroblasts were found in the lesion site rather than the surrounding parenchyma, but a number of those cells were often found in the meninges outside of the spinal cord as well; suggesting that transplant-derived fibroblasts may have survived and migrated out of the spinal cord in some cases (data not shown). In contrast, regardless of source, transplanted SCs were largely found surrounding the edges of the cavity, particularly within the spared rim of tissue at the margin of the spinal cord (arrows in Fig. 3.4A – SKP-SC & N-SC images and Fig. 3.4F’,F’’; also visible in Fig. 3.4L-L’’ and Fig. 3.4M-M’’) or within tissue bridges spanning the lesion site (arrowheads in Fig. 3.4A – SKP-SC & N-SC images and Fig. 3.4F’,F’’; also visible in Fig. 3.4L-L’’ and Fig. 3.4M-M’’). In summary, despite rather limited survival of transplanted SCs in the present study, SKP-SCs and N-SCs both showed very similar graft morphology and demonstrated better survival than transplanted fibroblasts in the injured cervical spinal cord (Fig. 3.4D).

### 3.3.5 Transplanted and endogenous SCs myelinate host axons after cervical SCI

SKP-SCs are known to myelinate axons in typical SC fashion – one SC per myelinated axon segment – and produce bona fide peripheral myelin as indicated by the presence of $P_0$,
appropriate distribution of nodal proteins (e.g., Caspr and Kv1.2) and confirmation of appropriate ensheathment and compact myelin formation using electron microscopy (Chapter 2; Biernaskie et al., 2006). Here, we found evidence of $P_0$-myelination in the injured cervical spinal cord for both the SKP-SC (arrowheads in Fig. 3.4N,P) and N-SC groups (arrowheads in Fig. 3.4O,Q), with the majority of this myelination occurring in rostral-caudal orientation throughout the spared rim and mid-lesion tissue bridges. Transplanted SCs in both groups showed similar myelination potential, as there was no significant difference between SKP-SC and N-SC transplanted rats with respect to the percent of GFP$^+$ area containing $P_0^+$ myelin structures (31 ± 7% and 26 ± 2%, respectively). Importantly, these numbers are in line with the estimate based on cell counts in our previous work (~35% in Chapter 2). Intermingled with the transplant-derived myelinating SCs we found a number of endogenous SCs (arrows in Fig. 3.4N) as well as non-myelinating ($P_0^- / p75^+$) transplant-derived (GFP$^+$) SCs (arrows in Fig. 3.4O-Q; * in 4N,O) in both SKP-SC and N-SC treated spinal cords.

In previous work transplanting SKP-SCs we noted that endogenous myelinating SCs accounted for approximately half (53%) of the $P_0^+$ structures in the contused thoracic spinal cord, and that those structures occupied substantial regions entirely devoid of GFP (Chapter 2). In contrast, here we saw that the majority of $P_0^+$ structures found in SKP-SC or N-SC transplanted spinal cords after cervical crush and acute transplantation were in regions occupied by transplant-derived GFP$^+$ cells (Fig. 3.4L-L'' for SKP-SC, Fig. 3.4M-M'' for N-SC). As an estimate of the contribution of endogenous SCs, we compared the percent of $P_0^+$ area that was also GFP$^+$ in SC transplanted animals in Experiment 2 and found that only ~35-40% of the $P_0^+$ area was GFP$^+$ (SKP-SCs: 40 ± 6 %; N-SCs: 35 ± 3 %). These data indicate that the majority of
SC myelin (~60-65%) came from transplant-derived cells in both the SKP-SC and N-SC groups, and compared to our previous thoracic work, these results suggest a somewhat diminished endogenous SC response in the injured cervical spinal cord.

We found a substantial endogenous (P0+ / GFP+) population of myelinating SCs in fibroblast transplanted rats (Fig. 3.4G). These cells produced a total P0+ myelin volume that was not significantly different from that produced by transplanted and endogenous SCs in the SKP-SC and N-SC treated groups in Experiment 2 (data not shown). However, the SCs present in fibroblast treated tissue were nearly all restricted to the lesion site and showed a haphazard organization (Fig. 3.4G) rather than the rostral-caudal orientation associated with P0+ SC myelin in the spared rim and lesion bridges of SKP-SC and N-SC transplanted rats (Fig. 3.4L,M, respectively). In contrast, we found little P0+ myelin in media treated animals (data not shown), in line with previous evidence that the endogenous SC response is more robust in the presence of transplanted SKP-SCs and N-SCs (Chapter 2; Hill et al., 2006; Assinck et al., submitted).

3.3.6 SKP-SC transplantation does not increase reactive astrogliosis relative to media treatment after cervical SCI

In light of previous evidence that N-SCs enhance reactive astrogliosis after transplantation in the injured spinal cord (Pearse et al., 2004a), we assessed the degree of reactive astrogliosis by measuring immunoreactivity for GFAP (GFAP-IR) and the CSPG, neurocan (neurocan-IR), in tissue immediately adjacent to the lesion site at 11 wpi. Despite using similar histological methods on tissue from both experiments, we noted higher GFAP-IR values for animals in Experiment 1 versus Experiment 2 (Fig. 3.5C,D), which most likely resulted from variability in antibody labelling/penetrance between the two experiments. GFAP-IR and
Figure 3.5  SKP-SC transplantation does not increase reactive astrogliosis

A,B: Representative images of GFAP- (A) or neurocan- (B) immunolabelled longitudinal sections at the level of injury at 11 wpi for each group in Experiment 1 (Media & SKP-SC) and Experiment 2 (SKP-SC, N-SC, Fibro). In tissue immunolabelled for neurocan (B), dashed lines mark the boundary between gray matter (GM) and white matter (WM). C-F: GFAP- (C,D) and neurocan- (E,F) immunoreactivity (IR) data for individual animals with the group median depicted as a solid black line (–). There were no significant differences between the SKP-SC and media treated groups (Expt 1) on GFAP-IR or neurocan-IR measured rostral (C,E, respectively) or caudal (D,F, respectively) to the injury site. The same results were found for the groups in Experiment 2, except for GFAP-IR measured rostral to the injury site (region marked by arrowheads in A), where the N-SC treated rats showed significantly higher GFAP-IR than either the SKP-SC or Fibro groups (C). C-F: * p < 0.05.
neurocan-IR measures showed positive normalized values greater than 1 (Fig. 3.5C-F) in all animals, regardless of treatment; indicating that GFAP and neurocan production were upregulated in tissue adjacent to the lesion compared to contralateral uninjured tissue; as expected given that SCI alone is associated with glial scar formation and increased reactive astrogliosis (Fitch and Silver, 1997; Silver and Miller, 2004).

In Experiment 1, we found no significant difference between media and SKP-SC treated rats on GFAP-IR (Fig. 3.5A,C,D – Expt 1) or neurocan-IR (Fig. 3.5B,E,F – Expt 1) measured rostral or caudal to the injury site. In Experiment 2, comparing different types of cell transplants we found a significant difference among the treated groups in terms of normalized GFAP-IR rostral ($\chi^2(2)=6.44$, $p=0.040$), but not caudal to the lesion site (Fig. 3.5A,C,D – Expt 2). Follow-up pairwise testing revealed that the N-SC transplanted group had significantly higher rostral GFAP-IR than either the SKP-SC ($U_{(20)}=28.00$, $p=0.035$) or Fibro ($U_{(19)}=25.00$, $p=0.039$) treated groups (Fig. 3.5C). The latter finding was surprising in light of previous evidence that skin fibroblasts elicit more pronounced GFAP-IR than N-SCs after transplantation into the wire knife-lesioned lumbar dorsal column (Toft et al., 2013), but the numerous methodological differences between studies may explain that disparity. Curiously, this difference in GFAP production by astrocytes rostral to injury in response to N-SC transplantation did not translate into a significant increase in production of the CSPG neurocan, as neurocan-IR did not differ among the groups (Fig. 3.5B,E,F – Expt 2). Thus, the results of Experiment 1 indicated that SKP-SC transplantation does not elicit enhanced reactive astrogliosis compared to media treatment, whereas the results of Experiment 2 were ambiguous with respect to the relative degree of reactive astrogliosis elicited by SKP-SCs versus N-SCs; as although the N-SCs induced higher
GFAP expression rostral to the injury site they did not elicit increased CSPG expression in that region.

3.3.7 SC transplantation is associated with reduced RST atrophy and/or enhanced RST sparing/plasticity

To determine whether the cell transplants promoted sparing or regeneration of rubrospinal axons following cervical DLF crush injury, we injected the axonal tracer BDA into the red nucleus to anterogradely fill descending RST fibres. In the absence of RST axon sparing/regeneration in the white matter at the level of injury in any of the treatment groups, we speculated that changes in RST sparing/plasticity may have occurred in the gray matter, where RST collaterals branch extensively at all levels of the spinal cord (Küchler et al., 2002). To examine this possibility we chose to analyze BDA-labelled RST axon density (RST density) in the ipsilateral spared gray matter (GM) at C2 and far rostral (~1mm rostral), immediately rostral and immediately caudal to the injury site (as depicted in Fig. 3.6A); or C4/5 in the case of uninjured controls. Sample images depicting near-median BDA⁺ axon densities based on raw data from each group at each level of analysis revealed RST axons descending in the DLF of the white matter (WM), and innervating the GM at C2 (top row), far rostral (second row), rostral (third row), and caudal (bottom row) to C4/5 (i.e., the injury level) in both the uninjured control group and the injured/treated groups in each experiment (Fig. 3.6B). Due to systematic differences in BDA tracing efficacy, the results of the RST density analyses in Experiment 1 cannot be directly compared to those from Experiment 2, including the uninjured control group (see Methods), and so we conducted separate analyses of each and present our findings in this section accordingly.
Figure 3.6 SKP-SC transplantation increases rubrospinal axon density in the gray matter following incomplete cervical crush injury

A: Diagram depicting coronal section (at C2) and cervical region of the injured rat spinal cord following a left C4/5 DLF crush. The red area in the coronal section represents the RST, which was completely ablated by the injury at C4/5 in all tissue examined. The injury site (black) encompassed nearly all of the white matter (WM; white) and a portion of the gray matter (GM; gray) at C4/5. BDA-labelled RST axon counts or density were quantified at C2, far rostral (FR), immediately rostral (R), and immediately caudal (C) to the injury site (boxed regions). B: Sample images of BDA-labelled RST axons from coronal (C2) or longitudinal (Far Rostral, Rostral, Caudal) sections at 11 wpi for each group. C-L: RST axon counts/density data (Expt 1: C-G; Expt 2: H-L; group medians depicted as solid black lines [–]), including BDA⁺ axon counts in the WM at C2 (C, H) and normalized (Expt 1) or raw (Expt 2) RST axon density in the GM at C2 (D, I), far rostral (E, J), rostral (F, K), or caudal (G, L) to the lesion site, grouped according to injury/cellular transplantation (and CsA treatment in Expt 1 only; with CsA [+] and without CsA [–]). In the absence of CsA, the SKP-SC group had significantly greater RST axon density than media controls at all levels (D-G). In the presence of CsA, RST axon density in the GM was increased in the Media group (E, F) and decreased in the SKP-SC group (E-G). In Experiment 2, uninjured animals tended to have higher BDA⁺ axon counts at C2 and densities at all levels, but only differed significantly from SKP-SCs in terms of caudal RST axon density (L). Both uninjured controls and SKP-SCs had significantly higher RST axon density than the N-SC or Fibro groups in the GM at C2 (I), far rostral (J), and rostral (K) levels, whereas all of the injured/treated groups had significantly lower RST density than the uninjured group caudal to the injury (L). C-L: * p < 0.05, ** p < 0.01, *** p < 0.005.
In Experiment 1, the media and SKP-SC groups had similar numbers of BDA$^+$ RST axons in the WM, but the media group tended to have fewer BDA$^+$ RST axons in the GM at each level compared to the SKP-SC group, and both groups had very few BDA$^+$ RST axons caudal to the injury site (Fig. 3.6B – Expt 1 images). As the SKP-SC and Media groups did not differ significantly in terms of axon counts in the WM at C2 (data not shown), we normalized all of the RST density values at each level in subsequent analyses. Those analyses revealed that the SKP-SC group had significantly higher RST density in the GM at C2 ($U_{(22)}=23.00$, $p=0.008$), strong trends toward such a difference at far rostral and rostral levels of analysis ($U_{(22)}=35.00$, $p=0.053$ for both), but no such tendency caudal to the injury (data not shown). In support of the notion that SKP-SCs promote higher RST density in the GM rostral to injury, we found a significant positive correlation between normalized RST density at C2 and SKP-SC transplant volume in Experiment 1 (Spearman’s $\rho=-0.622$, $p=0.031$), irrespective of CsA administration. That correlation indicated that animals with better SKP-SC graft survival tended to have higher RST density in the GM at C2, regardless of CsA administration, as the latter did not improve SKP-SC graft survival in this study.

Next we examined whether CsA administration influenced the RST density measures, and although there were no significant differences in the axon counts at C2 among the groups regardless of transplant or CsA treatment (Fig. 3.6C), we found that CsA administration significantly altered RST density measures in both the media and SKP-SC treated groups (Fig. 3.6D-G) at far rostral ($\chi^2_{(3)}=13.68$, $p=0.003$), rostral ($\chi^2_{(3)}=13.36$, $p=0.004$), and caudal levels ($\chi^2_{(3)}=12.74$, $p=0.005$), and very nearly did so at C2 as well ($\chi^2_{(3)}=7.63$, $p=0.054$). Media treated rats administered CsA generally had higher RST densities than those that did not get CsA, and
this difference reached significance at far rostral and rostral levels of analysis ($U_{(7)}=0.00$, $p=0.014$ for both; Fig. 3.6E,F). Surprisingly, the SKP-SC group showed the opposite tendency, as CsA treated rats had RST densities that did not differ significantly from those of rats treated with media and CsA (Fig. 3.6D-G), but those densities were significantly lower than rats treated with SKP-SCs in the absence of CsA on far rostral, rostral and caudal measurements ($U_{(13)}=8.00$, $p=0.021$; $U_{(13)}=10.00$, $p=0.037$; and $U_{(13)}=3.00$, $p=0.004$; respectively; Fig. 3.6E-G). These results suggest that CsA improved RST density in the GM of media treated animals up to at least 1mm rostral to the lesion following injury, but impaired the benefits associated with SKP-SC transplantation at every level below C2. Importantly, the effects of CsA cannot be explained in terms of a difference in SKP-SC survival, as graft volume was not significantly altered by CsA administration in this experiment.

The fact that CsA administration had opposite effects in animals treated with media versus those treated with SKP-SCs appeared to play a major role in the outcome of the RST analyses, as in the presence of CsA, RST density did not differ significantly between the SKP-SC and media groups at any level (Fig. 3.6D-G), but in the absence of CsA, the media group had significantly lower GM RST densities than the SKP-SC group at every level (C2: $U_{(9)}=3.00$, $p=0.038$; far rostral and rostral: $U_{(9)}=0.00$, $p=0.008$ for both; caudal: $U_{(9)}=1.00$, $p=0.014$; Fig. 3.6E-G). We found no significant difference between media and SKP-SC treated groups with respect to GM sparing adjacent to the lesion in this experiment, regardless of CsA treatment (data not shown), so these effects cannot be simply explained in terms of enhanced neuroprotection of the GM.
In Experiment 2, all of the animals were administered CsA to prevent immune rejection of the transplanted cells, as the analysis of Experiment 1 data had yet to reveal that CsA did not improve cell survival following acute transplantation, or that CsA may impair the ability of transplanted cells to improve RST density in the GM following injury. As such, CsA treatment could not be included as a variable in analyses of RST densities in Experiment 2. The results of Experiment 2 demonstrated that compared to uninjured control animals, injured animals had fewer BDA$^+$ RST axons in the WM ($U_{(28)}=17.00, p=0.004$) and lower GM RST density every level assessed (C2: $U_{(33)}=30.00, p=0.013$; far rostral: $U_{(33)}=16.00, p=0.002$; rostral: $U_{(33)}=20.00, p=0.003$; caudal: $U_{(33)}=1.00, p=0.000$), and this was particularly obvious caudal to C4/5 (i.e., caudal to injury) (Fig. 3.6B – Expt 2 images; data not shown). However, the number of BDA$^+$ RST axons at each level also appeared to differ among the three treatment groups, as animals in the SKP-SC group tended to have more BDA$^+$ RST axons than animals in the N-SC or Fibro groups at all levels rostral to injury, whereas animals in the N-SC group appeared to have a slight advantage over the other two groups caudal to injury (Fig. 3.6B – Expt 2 images). Preliminary analysis of the RST axon counts in the WM at C2 revealed significant differences among the groups traced in Experiment 2, including the uninjured controls ($\chi^2_{(3)}=7.77, p=0.020$; Fig. 3.6H). Follow-up testing demonstrated that axon counts were significantly higher in the uninjured control group than the N-SC or Fibro groups ($U_{(13)}=9.00, p=0.034$ and $U_{(12)}=6.00, p=0.002$, respectively), and that measure was also significantly higher in the SKP-SC group compared to the Fibro group ($U_{(13)}=2.00, p=0.003$).

These results cannot be explained in terms of differences in BDA tracing efficacy, as all of the animals from those groups were traced in random order over the same days, using the
same batches or BDA injected by the same person. These effects were also unlikely to be due to RST dieback, as we found that dieback failed to reach C2 in our previous work using the C4/5 DLF crush injury model in SD rats (Bretzner et al., 2008). Given that injury is known to induce substantial atrophy of RST neurons (Kobayashi et al., 1997; Kwon et al., 2004), and that the uptake and axonal transport of BDA are active processes that require energy (Glover et al., 1986; Terasaki et al., 1995; Dai et al., 1998), it stands to reason that atrophied axonal tracts would fail to transport BDA, and this has been shown to be the case following SCI in previous work (Lasiene et al., 2008). As such, the differences in BDA+ RST axon counts among the groups provide evidence for differential RST atrophy following injury and treatment in Experiment 2. In light of that, we could not normalize the RST density measures to the axon counts in Experiment 2 without eliminating potentially meaningful differences among the groups, so we conducted our analyses of RST densities in the GM at each level using the raw data in that experiment.

The raw RST densities in the GM differed significantly among the groups in Experiment 2 at all levels of analysis (C2: \( \chi^2 (3) = 15.76, p = 0.001 \); far rostral: \( \chi^2 (3) = 18.11, p = 0.000 \); rostral: \( \chi^2 (3) = 16.83, p = 0.001 \); caudal: \( \chi^2 (3) = 17.01, p = 0.001 \); Fig. 3.6I-L). Follow-up testing revealed that the SKP-SC group did not differ significantly from the uninjured group at any level of testing rostral to the injury, whereas the N-SC and Fibro groups both had significantly lower RST densities than the uninjured group at C2 (\( U_{(14)} = 6.00, p = 0.009 \) and \( U_{(13)} = 0.000, p = 0.001 \), respectively), far rostral (\( U_{(14)} = 2.00, p = 0.002 \) and \( U_{(13)} = 1.00, p = 0.002 \), respectively) and rostral (\( U_{(14)} = 4.00, p = 0.005 \) and \( U_{(13)} = 2.00, p = 0.003 \), respectively) levels of measurement (Fig. 3.6I-K). In addition, the SKP-SC group also had significantly higher RST densities than the N-SC and Fibro groups at those same levels (C2: \( U_{(18)} = 20.00, p = 0.023 \) and \( U_{(17)} = 11.00, p = 0.006 \); far
rostral: $U_{(18)}=16.00$, $p=0.010$ and $U_{(17)}=11.00$, $p=0.006$; rostral: $U_{(18)}=17.00$, $p=0.013$ and $U_{(17)}=11.00$, $p=0.006$; all for N-SC and Fibro groups respectively; Fig. 3.6I-K). In contrast, all of the injured groups showed a large and significant decline in RST density in the GM caudal to injury compared to the analogous level of assessment (caudal to C4/5) in the uninjured controls (SKP-SC: $U_{(14)}=0.00$, $p=0.001$; N-SC: $U_{(14)}=1.00$, $p=0.002$; Fibro: $U_{(13)}=0.00$, $p=0.001$), and here it was the N-SC group that appeared to have an advantage, as 4/10 of the N-SC treated animals had higher RST densities than any of the animals in either the SKP-SC or Fibro groups caudal to injury, although the differences among the three treatment groups failed to reach significance at that level (Fig. 3.6L).

With respect to the functional relevance of these effects, it is of interest to note that we found a significant positive correlation between CatWalk Intensity measured at 10 wpi and both WM axon counts at C2 (Pearson’s $r=0.43$, $p=0.035$) and raw RST density in the GM caudal to the lesion (Spearman’s $\rho=0.474$, $p=0.009$) among the injured groups in Experiment 2. Those correlations indicated that animals with more BDA$^+$ RST axons at C2 or higher RST density caudal to injury placed more weight on the forepaw (left) affected by injury. Although the N-SC group did not differ significantly from the Fibro group at any level, it is important to note that both SC groups had a number of animals that had higher axon counts than any animal in the Fibro group, and the N-SC group also had 4 animals with much higher gray matter RST densities caudal to injury; which nearly resulted in a significant difference between the N-SC and Fibro groups ($U_{(17)}=23.50$, $p=0.078$), but not the N-SC and SKP-SC groups ($U_{(18)}=34.00$, $p=0.225$) at that level. These results suggest that higher axon counts at C2 (i.e., less RST atrophy) or higher RST density caudal to injury in SC transplanted animals may both contribute to the functional
benefits associated with SC transplantation after SCI. Among the injured/treated animals in Experiment 2 we also noted a significant negative correlation between distal motor threshold and raw RST density far rostral and rostral to injury (Spearman’s $\rho=-0.571$, $p=0.0005$ and Spearman’s $\rho=-0.558$, $p=0.007$, respectively); indicating that animals with higher RST density in the GM up to 1mm from the lesion required less stimulation to elicit motor responses in the distal forelimb affected by injury. Those correlations suggest that enhanced RST sparing/plasticity contributed to improved RST electrophysiological functions, particularly for the SKP-SC group, which had significantly higher RST sparing/plasticity than either other group at those levels.

3.3.8 SC transplants preserve the spared tissue rim and mid-lesion bridges, providing a substrate for spared and sprouting axons

In the present study we measured the width of the spared rim immediately lateral to the injury site in 2 sections near the lesion epicentre following treatment of a DLF crush at C4/5. With the exception of the fibroblast group, we noted that the groups in Experiment 2 tended to have thicker spared rim widths than the groups in Experiment 1 (compare Expt 1 and Expt 2 data in Fig. 3.7A,B); which illustrates that differences exist between the two experiments despite our best attempts to use precisely the same methods. Although wider spared tissue rims tended to be found in the SKP-SC treated group, the results of Experiment 1 showed no significant difference between SKP-SC and media treated groups overall (Fig. 3.7A,B – Expt1). It should be noted that this analysis did not include spared mid-lesion tissue bridges, as those structures tend to be found in ventral sections, not near the lesion epicentre, where this analysis was conducted. In Experiment 2, we found a significant difference among the three treatment groups ($\chi^2(2)=10.29$,
$p=0.006$), as both SC groups had significantly larger spared rim widths than the Fibro group (SKP-SC: $U_{(13)}=5.00, p=0.006$; N-SC: $U_{(14)}=8.00, p=0.009$; Fig. 3.7B – Expt 2), which generally lacked any rim of spared tissue at the centre of the lesion (Fig. 3.7A – Expt2).

In SKP-SC and N-SC treated tissue, NF/βIIIItub-positive axons were found traversing both the spared rim (arrows in Fig. 3.7C) and tissue bridges spanning the lesion site (arrowheads in Fig. 3.7C). Whether these axons were spared or regrew after injury is unknown, but the fact that SC treated animals showed preservation of mid-lesion tissue bridges and spared tissue rims compared to fibroblast treated animals indicates that SC transplantation preserves more substrate capable of supporting axons that are oriented appropriately for the formation of functional connections across the injury site. In sharp contrast, the NF/βIIIItub-positive axons in fibroblast treated animals that lacked a lesion cavity were clearly oriented towards the centre of the lesion (* in Fig. 3.7C) and/or laterally towards the outside of the spinal cord. As a result, the arrangement of axons in the fibroblast transplanted animals seems unlikely to yield any functional benefit regardless of the degree of axonal growth promoted by the cells, as the axons appear to either stop inside the lesion, or turn perpendicular to the length of the cord towards the periphery – neither of which facilitates the reformation of functional connections between the parenchyma on each side of the lesion.

A similar pattern of growth/sparing was also observed for TH- and SERT- positive axons in the Fibro group (Fig. 3.7D and 7D’, respectively). TH is a marker for noradrenergic fibres, whereas SERT labels serotonergic fibres, and given that those fibre populations primarily arise from the brainstem (locus coeruleus and raphe nuclei, respectively) in the uninjured spinal cord (Ramon-Cueto et al., 1998; Greene, 2006), it seems likely that some portion of those fibres are
Figure 3.7  Compared to fibroblasts, transplanted Schwann cells enhance the amount of substrate available for axons to traverse the lesion from rostral to caudal

A: Representative images (based on group median spared rim widths) of GFAP-immunolabelled tissue at the level of injury, depicting the relative sizes of the spared tissue rims (arrows) in each treatment group. B: Spared rim width (µm) depicted as individual data points with group medians indicated by solid black lines (−);*** p < 0.005. Both SC groups had significantly thicker spared rims than the Fibro group (Expt 2), as the majority of animals in the latter group had no spared rim at the lesion epicentre (A,B). C: Sample images of NF/βIIIItub⁺ axons in each group in Experiment 2. Axons were oriented rostro-caudal in the spared rim (arrows) and mid-lesion bridges (arrowheads) of N-SC and SKP-SC treated tissue, whereas axons within the lesion site of animals in the Fibro group were oriented towards the middle of the lesion (*) in C) and/or the lateral edge of the spinal cord (i.e., the periphery). D-I′: Sample images from animals in each group in Experiment 2 immunolabelled for GFP (green), GFAP (blue), and TH (red; D,E,F,G,H,I) or SERT (white; D′,E′,F′,G′,H′,I′). Similar to NF/βIIIItub⁺ axons (C), TH⁺ and SERT⁺ axon subpopulations both lacked appropriate rostral-caudal orientation in the Fibro group (D & D′), but crossed the spared tissue rim (arrows in E, E′ [N-SC] and G,H, G′,H′ [SKP-SC]) or mid-lesion bridges (arrowheads in G,H, G′,H′) in the SC groups. In the latter, axons were found in intact GFAP⁺ regions or regions occupied by GFP⁺ SCs (arrowheads in F′ [a higher magnification image of the box in E′]). The density of TH⁺ and SERT⁺ axons was highest rostral to the lesion and lowest caudal to the lesion in both SKP-SC (G, G′) and N-SC (E,E′) groups, and at the caudal extent of the lesion TH⁺ and SERT⁺ axons appeared to exit the spared rim (arrowheads in F,I,I′ [higher magnification images of the boxes in E,H,H′]) and the mid-lesion bridges (*) in G,H,G′,H′). All data from longitudinal tissue samples at 11 wpi.
descending supraspinal axons. These subpopulations of putative brainstem-spinal axons were both found in very high density on the rostral side of the lesion in all cell transplant groups (TH: Fig. 3.7D, E, G, H; SERT: Fig. 3.7D′,E′,G′,H′). They were also found throughout the spared tissue rims and lesion-spanning bridges in both N-SC (TH: Fig. 3.7E; SERT: Fig. 3.7E′ & 7F′; only spared rim depicted) and SKP-SC (TH: Fig. 3.7G & 7H; SERT: Fig. 3.7G′ & 7H′; both spared rim and mid-lesion bridge depicted) transplanted spinal cords, although there was a tendency for TH+ axons to be denser in the mid-lesion bridges and SERT+ axons to be denser in the spared rim (compare Fig. 3.7H to H′). The density of both TH+ and SERT+ axons declined near the caudal portion of the spared rim and tissue bridges, but a small number of fibres were observed on the caudal side of the lesion in all N-SC (TH: arrowheads in Fig. 3.7F; SERT: data not shown) and SKP-SC (TH: * in Fig. 3.7G & H, arrowheads in Fig. 3.7I, SERT: * in Fig. 3.7G′ & H′, arrowheads in Fig. 3.7I′) treated spinal cords we examined. Thus, the overall pattern of TH+ and SERT+ axon profiles was suggestive of descending, possibly supraspinal, axon growth through the spared rim and mid-lesion bridges.

In support of the notion that the size of this substrate somehow contributes to functional outcomes, we found a significant positive correlation between intact rim width and the intensity difference between the left and right forelimbs on the CatWalk in Experiment 2 (Spearman’s $\rho=0.523$, $p=0.013$). This correlation indicates that animals with larger spared rim widths put more weight on the forelimb affected by the injury. Importantly, this correlation was significantly stronger if we excluded the animals in the Fibro group, many of which had a spared rim width of zero (Spearman’s $\rho=0.731$, $p=0.005$); indicating that even among SC treated animals only, more tissue sparing is associated with improved forelimb function.
3.4 Discussion

Previously we demonstrated the efficacy of SKP-SCs as a treatment for thoracic spinal cord contusion using a delayed transplantation paradigm in rats (Chapter 2). Here we demonstrated the efficacy of acutely transplanted SKP-SCs as a treatment for partial cervical spinal cord crush and we demonstrated that both SKP-SCs and N-SCs are preferable to dermal fibroblasts as a therapy for SCI. Compared to media treatment (Expt. 1), SKP-SC transplantation improved forelimb behavioral and electrophysiological recovery and spared/stimulated more rubrospinal axon branches in the GM rostral and caudal to the lesion site (particularly in the absence of CsA). Compared to dermal fibroblast transplantation (Expt. 2), treatment with SKP-SCs or N-SCs improved forelimb locomotor and electrophysiological functions, decreased RST atrophy, and/or the sparing/plasticity of RST axon branches in in the GM ipsilateral to injury, and enhanced sparing of the WM tissue rim at the edge of the spinal cord adjacent to the lesion site. Although SKP-SCs and N-SCs had largely similar morphological and functional effects, there were some differences between the two, as SKP-SCs induced less GFAP in reactive astrocytes rostral to injury, elicited greater RST sparing/plasticity in the GM rostral to injury, and improved electrophysiological latencies to levels not different from uninjured controls.

Compared to our previous transplantation of SKP-SCs at 7 days after thoracic contusion (Chapter 2), we found diminished survival of transplant-derived SKP-SCs and a reduced endogenous SC response after acute treatment of cervical SCI. Heightened cell death most likely occurred due to cytotoxicity of the acute transplantation environment (Hill et al., 2006), as the inclusion/exclusion of CsA had no effect on SKP-SC graft volume in Experiment 1; indicating that immune rejection was not a key factor in cell loss. These effects appeared to extend to SCs
from both sources, as SC graft volume and total SC content did not differ among the transplant groups in Experiment 2. In light of that compromised graft survival, the present work should be repeated using delayed transplantation to determine whether enhancing cell survival would further improve the efficacy of transplanted SCs or alter the results of our comparisons between the SKP-SCs and N-SCs. However, despite compromised graft survival, here we found evidence for repair and functional recovery following acute transplantation of both SKP-SCs and N-SCs into the partially injured cervical spinal cord of the rat.

Experiment 1 demonstrated a novel and unexpected interaction between cell/media treatment and CsA administration with respect to RST sparing/plasticity in the GM after cervical SCI. In addition to being a T-cell inhibitor, CsA binds to the mitochondrial permeability transition pore to prevent mitochondria-induced cell death (Bernardi et al., 1994; Hansson et al., 2003), and is known to be neuroprotective after a variety of neurological insults (Khaspekov et al., 1999; Scheff and Sullivan, 1999; Friberg and Wieloch, 2002). Consistent with CsA-induced preservation of injured RST axons, we found that CsA slightly elevated RST densities rostral to injury in media treated animals. However, in the absence of CsA, we noted significantly greater RST densities in SKP-SC treated animals at all levels; suggesting that CsA inhibited the response of injured RST axons to the transplanted SKP-SCs. Despite those effects, SKP-SC transplantation improved functional recovery in Experiment 1 regardless of CsA administration, indicating that enhanced RST sparing/plasticity cannot account for all of the benefits conveyed by transplanted SKP-SCs. Although CsA administration had no significant influence on functional outcomes here, these findings have implications for the use of immunosuppression in pre-clinical experiments and the potential clinical application of non-autologous cell transplants.
that require immunosuppression. Whether similar effects occur for other cell types or other immunosuppressant drugs remains an interesting question for future investigation, as does the question of whether SKP-SCs and N-SCs respond differently to immunosuppressive drugs.

Experiment 2 demonstrated that injury is associated with increased RST atrophy, as indicated by a decline in the number of descending RST axons that label with BDA, as well as a significant decline in the RST density at every level of the GM assessed. Subsequent analysis revealed that SKP-SC transplantation prevented/reversed RST atrophy and the decline in RST density in the GM rostral to injury, as unlike the N-SC and fibroblast groups, the SKP-SC group was only significantly lower than the uninjured controls on RST density in the GM caudal to injury. Although direct comparisons of the groups in Experiment 1 and the uninjured group were precluded here by a significant difference in BDA tracing efficacy between the two experiments, similar effects were noted following injury in Experiment 1, particularly with respect to the loss of GM RST branches caudal to injury. The lack of a difference between media and SKP-SC groups in terms of RST atrophy in Experiment 1 is curious in light of the significant difference between the SKP-SC and fibroblast treated animals in Experiment 2, and suggests that the injured RST may respond better to media than fibroblast treatment.

Experiment 2 also demonstrated that SKP-SC or N-SC transplantation were associated with less RST atrophy and greater RST densities in the GM rostral or caudal to injury compared to fibroblast transplantation. Although those differences only reached significance for SKP-SC transplanted animals, axon counts and caudal RST densities both significantly correlated with behavioral and/or electrophysiological functions, suggesting that these effects contributed to the functional improvements for both types of SCs. In addition, both SKP-SCs and N-SCs induced
significantly more WM sparing compared to transplanted fibroblasts, and these effects also correlated with behavioral outcomes. Although fibroblast transplantation was associated with reduced cavitation, the filling of the lesion appeared to damage the spared rim and mid-lesion bridges, thereby eliminating appropriate substrates for spared/sprouting axons to traverse the lesion in a rostral-caudal orientation conducive to the maintenance/formation of functional connections across the injury site. Whether the axons observed traversing those structures in SC-transplanted animals were spared or growing fibres remains unclear, but the change in density of SERT- and TH-positive axons from rostral to caudal is suggestive of descending growth of those putative supraspinal brainstem axon populations.

Experiment 2 also provided the first side-by-side comparison of SKP-SCs and N-SCs transplanted into the injured CNS. In the absence of robust cell survival, neither cell type elicited regeneration of the injured RST following acute transplantation, nor were there any significant differences between transplanted SKP-SCs and N-SCs with respect to graft survival, neuroprotection (lesion/cavity volume or spared rim width) or myelination. In line with the notion that SKP-SCs may interact better with host astrocytes, we found that GFAP expression rostral to the lesion was significantly higher in the N-SC group, but this difference did not translate into decreased expression of the inhibitory CSPG, neurocan in tissue transplanted with SKP-SCs as opposed to N-SCs, and other signs of enhanced reactive astrogliosis (e.g., increased inhibition of axonal growth/sprouting in that region and decreased SC migration into host tissue [see Chapter 4]) were absent as well. Thus, the present findings failed to demonstrate a clear advantage for SKP-SCs over N-SCs in terms of the degree of reactive astrogliosis neonatally-derived cells induce in the injured spinal cord, but suggested that transplanted N-SCs may cause
astrocytes rostral to the injury site to enhance their expression of GFAP. SCs from both neonatal sources also appeared to support similar levels of growth/sparing of SERT- and TH- positive axons at the level of the lesion, which also contradicts evidence from our previous work that suggested that SKP-SCs have an advantage over adult N-SCs in that regard (Chapter 2).

In contrast, we did find evidence that SKP-SCs have an advantage over the N-SCs with respect to their ability to elicit higher RST sparing/plasticity in the GM at all levels rostral to injury. However, the only RST measures that correlated with behavioral recovery were the axon counts at C2 and the RST density caudal to injury; where no significant differences were found directly between the SKP-SC and the N-SC groups. Increased RST density up to 1mm rostral to injury did correlate with lower motor thresholds required to elicit activation of the distal musculature, and although the SKP-SCs promoted greater RST sparing/plasticity at those levels, motor thresholds did not differ significantly between SKP-SC and N-SC groups. In summary, despite discrete neuroreparative and electrophysiological advantages, transplanted neonatal SKP-SCs and N-SCs elicited largely similar repair processes and promoted the same degree of functional recovery after incomplete cervical SCI, and compared to adult N-SCs, neonatal N-SCs appeared to differ less from neonatal SKP-SCs in terms of their interactions with astrocytes or their ability to support axonal growth at the level of the lesion.

The distance between the transplanted cells and the RST neurons/axons showing sparing/plasticity in these experiments suggests that the cells likely exert their effect on the RST via the production of diffusible signals such as neurotrophic factors or cytokines. Both N-SCs and SKP-SCs are known to produce a variety of such factors (Reynolds and Woolf, 1993; Grothe et al., 2000; Campana et al., 2006; Höke et al., 2006; Xu et al., 2013) and RST neurons are
known to respond to some of the molecules SCs produce (e.g., BDNF, NT-4/5, GDNF) (Kobayashi et al., 1997; Kwon et al., 2002b; Dolbeare and Houle, 2003; Kwon et al., 2007). Indeed, the effect of CsA on the RST response to transplanted SKP-SCs supports this notion, as CsA has been shown to decrease the expression of the neurotrophin receptor TrkB in the hippocampus and midbrain of rats (Chen et al., 2010). The elevation of neurotrophic factors and cytokines after SCI is also known to enhance the neuronal excitability of uninjured circuits (Kerr et al., 1999; Shu and Mendell, 1999; Arvanian and Mendell, 2001; Garraway et al., 2003), so the production of such factors by transplanted SCs may also underlie the electrophysiological effects we observed. In addition, trophic mechanisms may explain why N-SC treatment was associated with less RST sparing/plasticity and slightly poorer RST efficacy (i.e., longer latency) than SKP-SC treatment here, as neonatal SKP-SCs have been found to produce higher levels of some trophic factors (e.g., NT-3 and NGF) than neonatal N-SCs in culture (Walsh et al., 2009).

Cervical injuries account for ~60% of SCI in humans (NSCISC, 2012), and improved arm/hand function is a high priority for those people, as even small functional gains can have a profound effect on the performance of routine daily activities and quality of life (Anderson, 2004). Here, for the first time we demonstrated the efficacy of SKP-SCs as a treatment for cervical SCI, and we showed that SKP-SCs or N-SCs are preferable to dermal fibroblasts for those purposes. Contrary to the hypotheses based on our previous findings, neonatal SKP-SCs and N-SCs demonstrated similar efficacy here after acute transplantation in a model of partial cervical SCI. Although we did not find the anticipated advantages for SKP-SCs over N-SCs here, when both of those cell types were generated from neonatal tissue sources, it is important to note that our hypotheses were largely based on comparisons between rodent neonatal/adult SKP-SCs
and N-SCs harvested from adult peripheral nerve (Chapter 2; Assinck et al., submitted). As such, it is conceivable that SKP-SCs have advantages over adult N-SCs that they simply do not have over neonatal N-SCs; a notion that remains to be definitively tested in future work directly comparing SKP-SCs and N-SCs generated from adult tissue sources. Our findings here represent a promising beginning for the study of SKP-SCs as a treatment for cervical SCI, but future pre-clinical studies of these cells should use cervical contusion models and delayed transplantation to maximize clinical relevance. Although the injury/transplant paradigms used here are of limited clinical relevance, our results contribute to a growing body of evidence that SKP-SCs are a suitable alternative to N-SCs for therapeutic SCI applications; which is a clinically relevant issue given that N-SCs are presently being tested in SCI clinical trials (Saberi et al., 2008; 2011; Talan, 2012).
Chapter 4:

Schwann Cells Generated from Neonatal Rodent Skin-derived Precursors are Functionally Indistinguishable from Species- and Age-Matched Nerve-derived Schwann Cells

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3 A version of this chapter is in preparation for publication. Sparling JS, Plemel JR, Biernaskie J, Miller FD, Tetzlaff W. Schwann cells generated from neonatal rodent skin-derived precursors are functionally indistinguishable from species- and age-matched nerve-derived Schwann cells
4.1 Introduction

The transplantation of Schwann cells (SCs) holds considerable promise as a therapeutic approach for spinal cord injury (SCI), but the optimal source of those cells for clinical application remains a matter of debate, as SCs generated from peripheral nerve (N-SCs) and skin-derived precursors (SKP-SCs) have both demonstrated efficacy in preclinical animal models of thoracic and cervical injury (Chapter 2; Chapter 3; Barakat et al., 2005; Schaal et al., 2007; Assinck et al., submitted). N-SCs are harvested by excising peripheral nerve, which results in permanent nerve damage and related functional deficits when those cells are generated from autologous tissue for clinical application (Hood et al., 2009). In contrast, skin-derived precursors (SKPs) can be harvested from the dermis of humans and rodents via a relatively non-invasive skin biopsy (Toma et al., 2001; Toma et al., 2005), which carries no risk of permanent functional deficits, and those cells can be differentiated into Schwann cells (SKP-SCs) in vitro and expanded at high purity for the purposes of therapeutic transplantation (Biernaskie et al., 2006; McKenzie et al., 2006). Thus, it seems that SKPs have advantages over peripheral nerve as a source of autologous SCs, both in terms of the ease and safety of their harvest.

Our previous work transplanting neonatal/adult rodent SKP-SCs indicated that those cells elicit little reactive astrogliosis and migrate/integrate into astrocyte-rich host tissue surrounding the lesion site in the injured spinal cord (Chapter 2; Assinck et al., submitted); which are properties not typically associated with adult N-SCs transplanted under similar conditions (reviewed in Williams and Bunge, 2012). Indeed, both neonatal and adult N-SCs are well known to enhance reactive astrogliosis and display poor migration and integration into astrocyte-rich territory in vitro and in vivo (Fishman et al., 1983; Sims et al., 1999; Wilby et al.,
1999; Iwashita et al., 2000; Lakatos et al., 2000; Lakatos et al., 2003; Fairless et al., 2005; Andrews and Stelzner, 2007; Afshari et al., 2010a; Afshari et al., 2010b). The response of resident astrocytes to transplanted N-SCs is thought to play a substantial role in limiting the regenerative potential of those cells in the injured CNS (Plant et al., 2001; Lakatos et al., 2003; Adcock et al., 2004; Afshari et al., 2010b), which may also explain why the SKP-SCs appear to support more axonal growth across the glial scar and into spared host tissue when transplanted into the contused thoracic spinal cord of the rat (Chapter 2). Support for that notion comes from the fact that other cell types that are known to induce little reactive astrogliosis and integrate well with astrocyte-rich spared host tissue (e.g., olfactory ensheathing cells [OECs], Schwann cell precursors [SCPs], and even N-SCs genetically modified to express polysialylated neural cell adhesion molecule [PSA-NCAM]) have also been associated with enhanced axonal growth across the distal graft-host interface (Ramer et al., 2004c; Richter and Roskams, 2007; Woodhoo et al., 2007; Agudo et al., 2008; Ghosh et al., 2012). Thus it would also appear that the SKP-SCs may have advantages over their nerve-derived counterparts, in terms of their suitability for transplantation into the injured CNS.

Here we tested that hypothesis, by examining the interactions of neonatal rat SCs, generated from backskin SKPs or peripheral nerve, with astrocytes both in vitro and in vivo. In particular, we compared SKP-SCs and N-SCs in terms of the degree of reactive astrogliosis they elicit from astrocytes, and their ability to migrate/integrate into astrocyte rich domains both in vitro and after transplantation into the injured cervical spinal cord. Given that SKP-SCs are newly generated cells differentiated from dermal precursors, we previously speculated that those cells may possess a less mature phenotype, more similar to that of SCPs or immature SCs, than
the dedifferentiated N-SCs that are isolated from peripheral nerve (Chapter 2); a notion supported by subsequent evidence that SCPs also interact more favourably than N-SCs with astrocytes in the injured/demyelinated CNS (Woodhoo et al., 2007; Agudo et al., 2008). We tested that hypothesis in the present work as well, by comparing cultured SKP-SCs and N-SCs in terms of their expression of a variety of proteins and genes known to be associated with specific stages of SC development. In light of previous evidence, we speculated that neonatal SKP-SCs possess a less mature SC phenotype than neonatal N-SCs, and we predicted that the SKP-SCs would induce less reactive astrogliosis and display a greater capacity to migrate and integrate into astrocyte-rich territory both in vitro and in vivo as a result.

4.2 Materials and methods

All experimental procedures used in this work were approved by the Animal Care Committee of the University of British Columbia and/or the Hospital for Sick Children Research Institute in accordance with the guidelines of the Canadian Council on Animal Care. Neonatal wildtype Sprague Dawley (SD) rats (P1-2; UBC Animal Care Facility, Vancouver, BC) served as the source of all tissue used to generate cells for the in vitro assays conducted in this work, whereas neonatal transgenic SD rats expressing green fluorescent protein (GFP; National Bio Resource Project Kyoto) served as the source of tissue to generate the GFP-positive (GFP⁺) cells used for the in vivo transplantation work; which included 22 adult male wildtype SD rats (Charles River, Wilmington, MA) that were injured and received cell transplants.
4.2.1 Cell culture for in vitro assays

4.2.1.1 Isolation of SKPs

As described previously (Biernaskie et al., 2006), back skin was dissected, minced, and digested for with collagenase type XI (Sigma; St. Louis, MO) to dissociate single cells. The suspension was passed through a 40µm cell strainer to remove undigested clumps of tissue and the resulting single-cell suspension was resuspended at an initial density of 50,000 cells/ml in SKP proliferation medium consisting of DMEM/F12 (3:1), 2% B27 supplement, 100U/ml penicillin, 100µg/ml streptomycin, 40µg/ml fungizone (Gibco/Invitrogen; Carlsbad, CA), 40ng/ml basic fibroblast growth factor, and 20ng/ml epidermal growth factor (both from BD Biosciences; San Jose, CA) and plated in T75 cell culture flasks (BD Biosci.) for expansion.

4.2.1.2 Differentiation of SKP-SCs

The protocol followed to differentiate SKP-SCs for use in the in vitro assays was modified from that previously described (Biernaskie et al., 2006). Briefly, primary or secondary SKP spheres were digested with collagenase for 15 minutes at 37°C and triturated to single cells prior to plating in 10cm tissue culture dishes (Sarstedt; Nümbrecht, Germany) coated with laminin (20µg/ml; BD Biosci.) and poly-D-lysine (PDL; 200µg/ml; Sigma) in SC proliferation media containing DMEM/F12 (3:1), 2% N2 supplement, 100U/ml penicillin, 100µg/ml streptomycin (all from Gibco), 50ng/ml neuregulin-1β (heregulin-β1; R&D Systems; Minneapolis, MN) and 5 μM forskolin (Sigma). The media was changed every 3-4 days and the cells were passaged once the dishes reached confluence.
4.2.1.3 Isolation of nerve-derived Schwann cells

The isolation and expansion of N-SCs for use in the in vitro assays described herein followed a protocol modified from that described in Chapter 3. Briefly, N-SCs were isolated from neonatal wildtype SD rat sciatic nerve segments by digestion in collagenase type XI at 37°C for 30-60 minutes. Cells were liberated by gentle trituration, and the dissociated cells were plated on 10 cm dishes coated with laminin/PDL and grown in precisely the same SC proliferation media as that used to grow the SKP-SCs (described above), with media changes every 3-4 days and passaging at confluence.

4.2.1.4 SC purification and expansion for assays

Both SKP-SCs and N-SCs were purified during passage 1 or 2 by switching media to DMEM containing 20% fetal bovine serum (FBS; PAA Labs; Toronto, CA) to preferentially promote expansion of contaminating fibroblasts, then applying 2-3 antimitotic pulses (10 μM each of uridine and 5′-fluoro-2′-deoxyuridine; both from Sigma; 2 days on followed by 2 days off). This method varies from the mechanical isolation with cloning cylinders that has been used to purify SKP-SCs in all previous work, and was tested here in an effort to increase the yield of SKP-SCs following the initial plating of the cells in the presence of SC mitogens and to ensure that SKP-SCs and N-SCs were purified using the same procedures. Following purification the SCs were removed from the dish, resuspended at 2 million cells/ml in 90% FBS and 10% dimethyl sulfoxide (DMSO; Sigma) and frozen at -80°C. SKP-SCs and N-SCs generated from tissue samples from four different animals were subsequently thawed and expanded further for plating in the various in vitro assays. For all experiments, SKP-SCs were grown in parallel with N-SCs, such that SCs from both sources were fed, passaged, purified, frozen, thawed and
expanded side by side using the same mixtures of reagents. Furthermore, an equal number of batches of SKP-SCs and N-SCs from the same passage number were used in each of the in vitro assays conducted for this work.

4.2.1.5 Isolation and expansion of astrocytes

In the present work, the isolation of astrocytes followed methods previously described to isolate astrocytes and oligodendrocyte precursors from the cortex (McCarthy and de Vellis, 1980; Dugas et al., 2006; Plemel et al., 2013). Briefly, the cortices of neonatal SD rats were isolated, diced and digested in papain (Worthington; Lakewood, NJ) at 37°C for 90 minutes then gently dissociated. Dissociated cells were resuspended in ‘astrocyte media’ containing high-glucose DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100U/ml penicillin, 100μg/ml streptomycin (all from Gibco) and 20% FBS. Cells were plated on PDL-coated T75 tissue culture flasks for 10-12 days. To purify astrocytes, confluent T75 flasks were shaken at 200rpm and 37°C for 19-21 hours to allow preferential detachment of microglia and oligodendroglia from the culture surface. Following shaking, the media (containing unwanted cells) was removed and replaced with fresh astrocyte media. To impede the proliferation of any remaining cellular contaminants, confluent cultures of primary astrocytes were pulsed (2 days on, 2 days off) with antimitotics (10 μM each of uridine and 5’-fluoro-2’-deoxyuridine) and to ensure maximal purity these cultures were shaken again immediately prior to passaging for use in the migration and boundary assays described below. To minimize the potential influence of variability among astrocyte cultures we used each batch of those cells in an equal number of assays with SKP-SCs and N-SCs simultaneously.
4.2.2 Cell culture for *in vivo* assay

The methods used to generate SKPs, differentiate SKP-SCs, and isolate N-SCs for the *in vivo* portion of the present work were described in detail in Chapter 3. In contrast to the cells used in the *in vitro* assays, all SKP-SCs and N-SCs were generated from neonatal transgenic GFP+ SD rat tissue for transplantation into the injured spinal cord. SKPs were isolated and expanded in the same manner described for the *in vitro* work, but for transplantation, SKP-SCs were initially differentiated from SKPs in the presence of SC proliferation media containing 10% FBS (for the first 3-5 days only), and the cells were purified by mechanically isolating patches of SC-like cells from contaminating cell types (e.g., fibroblasts) using cloning cylinders (Dow Corning; Midland, MI), rather than antimitotic treatments. N-SCs were expanded in SC proliferation media with added insulin (4µg/ml; Invitrogen) and (in some cases) 1% FBS, and the N-SCs grown for transplantation were not purified by antimitotics, but rather by sequential passaging under conditions promoting SC proliferation. Both SKP-SCs and N-SCs used for transplantation were fed using full media changes every 3-4 days and passaged 3-4 times prior to transplantation.

4.2.3 *In vitro* protein and gene expression analyses

4.2.3.1 Immunocytochemistry cultures

Approximately 125,000 passage 4 SCs from each batch were plated on laminin/PDL-coated 12mm coverglasses (0.13-0.17mm thick; Fisher Scientific; Waltham, MA) in 24-well tissue culture plates (BD Biosci.) with SC proliferation media (see above). Four days after plating these cells were fixed with 0.01M phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA), rinsed 2-3 times and stored in PBS (500 µl/well) at 4°C for subsequent
immunolabelling (see below). Four batches of SKP-SCs and four batches of N-SCs were compared in this work.

4.2.3.2 Western blot procedures

Approximately 4 million SCs from each batch of SKP-SCs and N-SCs were plated on laminin/PDL-coated 10cm culture dishes (Sarstedt) in SC proliferation media at passage 4. Protein lysates were collected from one 10cm dish of each SC batch 4 days after plating. Briefly, cells were rinsed with ice-cold Ca\(^{2+}\)/Mg\(^{2+}\)-free Dulbecco’s phosphate buffered saline (DPBS; Invitrogen), and 1ml of ice-cold RIPA lysis buffer (Rockland; Gilbertsville, PA) with protease inhibitors (Complete cocktail tablets; Roche; Laval, QC) was applied to each dish for 5 min. Lysed cells were scraped from the dish and the lysate was collected and frozen immediately at -80°C. After lysates were collected from all 8 SC batches, each samples was thawed, homogenized, and centrifuged (13,000 rpm for 20 min at 4°C) to remove particulate matter. Protein concentrations were determined using BCA protein titration (Pierce; Thermo Scientific, Rockford, IL), and 20 µg of protein from each sample were applied to separate lanes on the same gel and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by overnight electrophoretic transfer to Immobilon-P PVDF membranes (Millipore; Billerica, MA). Multiple gels were run to separate protein samples using those methods, with both reducing and non-reducing (i.e., in the absence of boiling or strong reducing agents such as β-mercaptoethanol) conditions being used to accommodate various antibodies and by varying the percentage of acrylamide used in the resolving gel for SDS-PAGE to accommodate the various sizes of our proteins of interest. Following transfer PVDF membranes were dried and stored at 4°C for subsequent immunoblotting.
For immunoblotting, membranes were washed in Tris-buffered saline (Fisher Scientific) containing 0.1% Tween-20 (Calbiochem; Merck, Darmstadt, Germany) (i.e., TBST), then incubated on a shaker for 1hr at room temperature in TBST with 5% skimmed milk or 5% bovine serum albumin (BSA; Sigma-Aldrich) to block non-specific binding of monoclonal and polyclonal antibodies, respectively. Membranes were rinsed with 0.5% blocking solution (TBST with 0.5% milk or BSA as appropriate) and incubated with primary antibodies diluted in 0.5% blocking solution at 4°C on a rotating platform overnight. The following morning, membranes were washed with 0.5% blocking solution, incubated with peroxidise-conjugated secondary antibodies diluted in 0.5% blocking solution on a shaker for 1hr at room temperature and then washed again. Membranes were treated with ECL detection reagents (Amersham Biosciences, Arlington Heights, IL) and imaged on a BioSpectrum imaging system using VisionWorksLS software (UVP; Upland, CA). The following primary antibodies were used for immunoblotting in the present study: rabbit anti-glial fibrillary acidic protein (GFAP; 1:200; Dako; Carpentaria, CA), rabbit anti-Krox20 (1:500; Covance; Emeryville, CA), mouse-anti neural cadherin (NCad; 1:2000; BD Biosciences), mouse anti-rat p75 neurotrophin receptor (p75; 1:1000, Millipore), chicken anti-protein zero (P0; 1:2000; Aves Labs; Tigard, OR), mouse anti-actin (1:40,000; MP Biomedicals; Santa Ana, CA), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500; Santa Cruz Biotech.; Santa Cruz, CA). Secondary antibodies used in this study included: peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG and donkey anti-chick IgY (all 1:10,000; Jackson ImmunoResearch, West Grove, PA). Following immunoblots for proteins of interest, membranes were stripped by submersion in buffer containing 100mM β-mercaptoethanol, 2% SDS, and 62.5mM Tris-HCl for 30 min at 70°C prior to immunoblotting.
again using primary antibodies against loading control proteins (actin or GAPDH depending on the membrane in question).

Bands for each protein of interest were confirmed to occur at positions appropriate for the molecular weights of the proteins of interest prior to final analysis. Immunoblot images were processed and analyzed using ImageJ software (NIH; USA) to measure the optical density of each band and express that as a percent of the total density associated with all bands on each membrane. The percent value from each lane was then normalized to lane 1 on the membrane, and subsequently normalized to the percent value associated with its own loading control (actin or GAPDH), providing the normalized relative optical density values from each sample for each protein of interest. Those values were averaged across 4 samples from each type of SC in order to compare the levels of the various proteins of interest in SKP-SC and N-SC samples.

4.2.3.3 qPCR procedures

For all gene expression measurements, passage 4 SKP-SCs and N-SCs were scraped from 10cm culture dishes (Sarstedt) 4 days after plating in SC proliferation media. Total mRNA was isolated and purified from each sample using mirVana extraction kits (Life Technologies; Carlsbad, CA) and reverse transcribed into cDNA using Superscript III and Oligo (dT)20 primers (both from Invitrogen; Carlsbad, CA). Genes of interest included: GAPDH (reference gene), cadherin-19 (Cad-19), NCad, p75, S100β, inhibitor of DNA binding 2 and 4 (Id2 and Id4, respectively), paired box gene 3 (Pax3), galactocerebroside (GalC), P0, Krox-20, myelin basic protein (MBP), and cell division control protein 2 homologue (Cdc2). Primer sets for each gene of interest were designed using NCBI primer-BLAST (NIH, USA), purchased from Integrated DNA Technologies (Coralville, IA), and validated by measuring the size of PCR product as well
as PCR efficiency. All of the primer sets used in this work demonstrated equivalent PCR efficiency to the GAPDH ‘reference gene’ primers used to normalize gene expression results.

The following primers were used to measure gene expression in the present experiment:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACAGCAACAGGGTGGTGAC</td>
<td>TTTGAGGGTGCAGCGAACTT</td>
</tr>
<tr>
<td>Cad-19</td>
<td>AGCCAATGACGCAGATGACCCT</td>
<td>GGCTCCAGGCTGACCAAGCA</td>
</tr>
<tr>
<td>NCad</td>
<td>GCGGCTTTGCTTCAGGCATCT</td>
<td>TGGCCTTCGTGCAACGTCTT</td>
</tr>
<tr>
<td>p75</td>
<td>AGCCAGAGCCTGCACGACCA</td>
<td>TGCCATCACCCCTTGAGGGCCTG</td>
</tr>
<tr>
<td>S100β</td>
<td>AGTCCACACCCCAGTCCTCTCTGGA</td>
<td>CCGGAGGCTCCTGTCACCTTTTG</td>
</tr>
<tr>
<td>Id2</td>
<td>ATGGAAATCCTGCACGACGTCA</td>
<td>ACGTTTGTTCTGTCCAGGTCTCT</td>
</tr>
<tr>
<td>Id4</td>
<td>ACTGTGTCCTCAGTCGATATGAA</td>
<td>TGCAGGATCTCCACTTTGTGCTGACT</td>
</tr>
<tr>
<td>Pax3</td>
<td>CGGCATTCGGCCTTGCGTCA</td>
<td>CAGGGCTTGTCACTTTGTGCTGG</td>
</tr>
<tr>
<td>GalC</td>
<td>ATGGCATCGGCAGTCAGGC</td>
<td>GGGAGGGTTCAGTGCCGTCTGT</td>
</tr>
</tbody>
</table>
Gene expression was measured using qPCR on a Viia7 Real-Time PCR System with Fast SybrGreen (both from Applied Biosystems; Carlsbad, CA) PCR product detection. Four samples of each cell type were used for all analyses except for measurements of Id2, Pax3, and Krox-20 which used 7 SKP-SC samples and 8 N-SC samples (1-2 samples each from 4 distinct tissue sources). All gene expression data were normalized to GAPDH expression levels and those normalized values were normalized again to the N-SC group average to provide a measure of comparative gene expression for analysis using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Normalized gene expression data were averaged over 4-8 samples per group (SKP-SC versus N-SC) for each gene of interest for subsequent graphical presentation and statistical analysis.

4.2.4 Inverted coverslip migration assay

We used an inverted coverslip migration assay, modified from that described by Fawcett and colleagues (Afshari et al., 2011), to assess SC migration over an astrocyte monolayer in
vitro. Briefly, ~180,000 passage 1 astrocytes were plated in PDL-coated Lab-Tek 2-well chambered coverglasses (Nunc; Thermo Fisher Scientific) and expanded in astrocyte media containing 10-20% FBS (see above) for 10 days to ensure that each well was completely confluent with astrocytes prior to running the assay. Meanwhile, 50,000 passage 4 SCs (SKP-SCs or N-SCs) were pre-plated 5-10 min on laminin/PDL-coated pieces of broken 12mm coverslips (Fisher) and grown in 12-well plates containing SC proliferation media for 24 hours. Next the SCs were labelled with CellTracker dye (CT dye; Invitrogen) following the manufacturer’s instructions. Briefly, the SCs were incubated in SC media containing CT dye for 30min, rinsed and incubated in fresh SC media for at least 30 min prior to coverslip inversion to start the migration assay. Coverslip fragments coated with CT dye-labelled SCs were rinsed repeatedly in warm, fresh DMEM to remove loose cells, then inverted and placed onto the astrocyte monolayers with the SCs facing down towards the astrocytes. Each fragment was applied to its own well and the media was replaced with SC proliferation media. Fifty hours after coverslip inversion the wells were fixed with 4% PFA in PBS, then rinsed and stored in PBS (2ml/well) at 4ºC for subsequent immunocytochemistry (see below). Cells were subjected to immunocytochemistry and all imaging procedures within the chambered wells, as the walls of the chambered coverslips could not be removed.

4.2.5 SC-astrocyte boundary assay

To examine the interaction between SCs and CNS astrocytes at boundaries between territories occupied by those two cell types, we conducted a SC-astrocyte boundary assay using techniques modified from those previously described (Afshari et al., 2011). Briefly, 50,000 SCs (SKP-SCs or N-SCs; passage 4) and 50,000 astrocytes (passage 2) were pre-plated for 30 min at
opposite ends of 4-well chambered culture slides (BD Biosciences) with a small gap left between the two cell types. These cultures were initially grown in SC proliferation media with an additional 10% FBS and fed using full media changes every 3 days, but under those conditions, we noted a lack of expansion by both SKP-SCs and N-SCs during the first week after plating. In contrast, SCs grown under the same conditions in the absence of astrocytes showed much better growth in the same timeframe (data not shown), indicating that the presence of astrocytes inhibited SC proliferation; as reported previously by (Guenard et al., 1994). This problem was alleviated by switching to half media changes every 2 days using DMEM/F12 (3:1) with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 100ng/ml neuregulin-1β, and 5μM forskolin. These cultures were maintained for 30 days, by which point the SC and astrocyte fields had made contact (i.e., formed boundaries) across the majority of the width of most wells. At that point, the cells in each well were fixed with 4% PFA in PBS, rinsed and stored in 1ml of PBS per well at 4°C for subsequent immunocytochemistry.

4.2.6 Spinal cord injury, cell transplantation, and tissue processing

To compare the in vivo migration/integration of SKP-SCs and N-SCs in astrocyte-rich territory we examined tissue from the transplantation experiment described in Chapter 3. Briefly, that study involved the transplantation of SKP-SCs and N-SCs generated from GFP+ neonatal rat tissues in a rat model of incomplete cervical spinal cord injury. The injury model used was a left cervical (C4/5) dorsolateral funiculus (DLF) crush, which was administered to anaesthetized adult male rats by crushing the left C4/5 DLF for 20s with custom-designed fine surgical forceps at a depth of 1mm. For transplantation, the SCs were resuspended in fresh DMEM at a density of 135,000 cells/µl and administered immediately after injury via three 0.5 µl stereotaxic injections
within the crush site (two medial injections at 0.5 and 1 mm depths, and one lateral injection at 0.5 mm depth) for a total of 1.5μl containing ~200,000 cells. All of the animals that received cell transplants were immunosuppressed with Cyclosporine A (CsA; Novartis Pharmaceuticals; Mississauga, ON) administered via daily injection (10 mg/kg per day, i.p.) or provided in homecage drinking water (Neoral, Novartis; 1.5 ml/L of water) for the duration of the experiment. Eleven weeks after injury and treatment the rats were sacrificed by lethal overdose and perfused transcardially with PBS, then 4% PFA in PBS. Cervical segments of spinal cord were dissected, post-fixed in 4% PFA in PBS overnight, cryoprotected in 24% sucrose in 0.1M phosphate buffer over 2-3 days, then frozen in isopentane over dry ice and stored at -80°C. Cervical segments from C3 to C6 were cut in 20μm-thick longitudinal sections in the horizontal plane using a cryostat, mounted on slides and stored at -80°C for subsequent immunohistochemistry.

4.2.7 Immunocytochemistry and immunohistochemistry

In vitro immunocytochemistry was conducted on cultured SKP-SCs and N-SCs to compare the two cell types with respect to the expression of known protein markers for SCs, to quantify SC purity and maturity and to analyze the results of the migration and boundary assays. Fixed cells were removed from storage, rinsed with PBS and incubated overnight at 4°C with PBS containing 10% normal donkey serum (Jackson ImmunoResearch), 10% BSA, 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and primary antibodies at appropriate dilutions. The next day, the cells were rinsed with PBS and secondary antibodies (diluted in PBS) were applied for 1 hour at room temperature, then rinsed and either left soaking in fresh PBS (2-well chambered coverglasses used in migration assay), mounted onto glass slides (24-well plate coverslips used
in purity and maturity assay), or coverslipped (4-well chambered slides used in boundary assay) prior to storage at 4°C until subsequent imaging. Triton X-100 was left out of the primary antibody solution used on cells labelled with CT dye (i.e., migration assays) and whenever O1 or O4 were the epitopes of interest (i.e., SC purity/maturity assays). The following primary antibodies were used for *in vitro* immunocytochemistry in the present study: mouse anti-rat p75 (1:400, Millipore), rabbit anti-p75 (1:300, Millipore), rabbit anti-GFAP (1:1000, Dako, Carpentaria, CA), chicken anti-GFAP (1:500, Millipore), mouse monoclonal anti-O1 (1:200, Millipore); mouse monoclonal anti-O4 (1:200, Millipore); mouse anti-S100β (1:400, Sigma). Secondary antibodies used for *in vitro* immunocytochemistry included: donkey anti-chicken, -mouse or -rabbit antibodies conjugated to Dylight 488 or 594 (1:300; Jackson ImmunoResearch). Nuclei were stained with Hoechst (1:5000; Invitrogen) during secondary antibody incubation.

Immunohistochemistry was conducted on frozen spinal cord sections thawed to room temperature for 30-60 minutes, rehydrated in PBS, and blocked for at least 10 minutes, then incubated for with 10% normal donkey serum containing 0.1% Triton for 30 min to block nonspecific binding. Chicken anti-GFP (1:1000; Chemicon, Temecula, CA) and rabbit (1:1000; Dako) or mouse (1:100; Sigma) anti-GFAP primary antibodies were diluted in 0.01M PBS containing 0.1% Triton and incubated with tissue overnight at room temperature. The next morning the tissue was washed and secondary antibodies (FITC-conjugated donkey anti-chicken and AMCA-conjugated donkey anti- rabbit or -mouse diluted in PBS at 1:200; Jackson ImmunoResearch) were applied for 2 hours at room temperature.
4.2.8 Image analysis, processing, and quantifications

Immunofluorescent images of cells and tissue were digitally captured using an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON, Canada) or a Zeiss AxioObserver Z1 confocal microscope fitted with a CSU-X1 spinning disc (Yokogawa Electric, Tokyo, Japan) and AxioVision 4.8 or Zen 2011 software (Zeiss). Digital images of cells and tissue were processed for presentation and/or quantification using Photoshop CS2 version 9.0 (Adobe Systems, San Jose, CA). SigmaScan Pro 5 (SPSS Inc., Chicago, IL) was used to measure areas and distances from images in all analyses.

4.2.8.1 Cell counts for SC purity and maturity analyses

SC purity was assessed by counting the number of p75\(^+\) cells sampled from 5 fields (>200 cells) per coverslip using confocal microscopy. Only healthy looking nuclei, as indicated by Hoechst staining, were counted and the resulting counts were converted to percentage of p75\(^+\) cells per coverslip. Two coverslips from each SC batch and 4 batches each of SKP-SCs and N-SCs were included in this analysis. SC maturity counts were conducted using the same sampling technique to count the number of p75\(^+\) cells with healthy nuclei that were also positive for O1 (O1\(^+\)), a marker of mature SCs in vivo. Maturity counts were conducted on 1-2 coverslips per SC batch, for all 4 batches of each SC type.

4.2.8.2 In vitro SC migration analysis

Confocal microscopy was used to count migrating SCs in each well of the inverted coverslip migration assay. The number of healthy (according to Hoechst nuclear stain), CT dye-labelled, p75\(^+\) SCs were counted in 0.3mm wide fields every 0.1mm out from the edge of the inverted broken coverslip up to 2mm away. Starting points at the edge of each side of a given
coverslip were selected at random by moving blindly to the centre of the coverslip fragment, then moving north, south, east, or west until the edge of the coverslip came in to view. Given the low number of migrating cells, these counts were subsequently summed to provide the total number of cells that had migrated between 0-0.1mm, 0.1-0.5mm, 0.5-1mm, and 1-2mm from the coverslip in any direction (i.e., north, south, east, or west). Wells where the broken coverslip moved at some point during the assay (indicated by damage to astrocytes or their absence along an edge of the coverslip fragment) were excluded from this analysis. The final counts included cells from all 4 batches of each SC type in 11 SKP-SC wells and 18 N-SC wells.

4.2.8.3  *In vitro* SC-astrocyte integration analysis

The integration of SCs into astrocyte-rich territory was assessed *in vitro* in SC-astrocyte boundary assays by quantifying the area occupied by p75⁺ SCs in the GFAP⁺ astrocyte territory in each well. The term ‘integration’ is used here to reflect the fact that the presence of SCs in astrocyte-rich regions is the result of migration by both cell types at the interface where they meet. Indeed, previous work using time-lapse observations of boundary assays found that SCs did not migrate into the astrocyte field, but rather were trapped there as they tried to retreat from the advancing astrocytes (Wilby et al., 1999). The integration area was normalized to the length of the border of contact between SCs and astrocytes in each well to account for well-to-well variability in the degree of SC-astrocyte contact. Three to four wells from each SC batch were quantified for this analysis, and group (SKP-SC versus N-SC) averages were subsequently calculated from the normalized values for each well.
4.2.8.4  *In vitro* GFAP-immunoreactivity

As a measure of astrocyte reactivity in the presence of SCs, immunoreactivity for GFAP (GFAP-IR) was quantified over confluent patches of cells on each side of the SC-astrocyte boundary by measuring the average intensity of the GFAP signal in three fields per well using SigmaScan Pro5. The values recorded on the astrocyte side of each well were normalized to those from the SC side of the same well to control for variability in immunocytochemistry between wells, and the resulting values were averaged by group. It should be noted that we initially normalized to GFAP-IR over astrocytes further from the border, but found nearly identical GFAP-IR values to those measured at the border. As such we chose to normalize astrocyte GFAP-IR to SC GFAP-IR in the same well because SKP-SCs and N-SCs were found to produce similar levels of GFAP by immunocytochemistry and Western blot (see Fig. 4.2). In the end, both normalization procedures produced the same results.

4.2.8.5  *In vivo* SC-astrocyte integration and SC migration analyses

We sampled from 3 levels within the lesion site (~320 µm apart) in 10 rats transplanted with SKP-SCs and 12 rats transplanted with N-SCs to generate data for all *in vivo* analyses. To examine the integration of transplanted SCs with GFAP⁺ astrocyte-rich host parenchyma we measured the total GFP⁺ pixel area found in regions of GFAP⁺ intact parenchyma outside of the lesion and averaged those 3 measures to get an individual ‘integration area’ for each animal. The *in vivo* migration potential of transplanted SCs was assessed by measuring the shortest possible distance between the edge of the lesion site and the single furthest SC from the lesion in each of the tissue sections used for the area analysis. Cells that had clearly migrated outside of the cord or through the central canal were ignored and the maximum migration distance for a single SC
from each animal was used in the final statistical analysis. Immunoreactivity for GFAP near transplant-derived SCs was assessed by measuring GFAP immunofluorescent signal intensity in a 50-pixel wide line through regions of host tissue adjacent to the lesion that contained or were immediately adjacent to GFP⁺ cells. GFAP-IR measures were normalized to the GFAP intensity measured in similar regions contralateral to the injury/transplant and averaged from three sections at the level of the lesion site to generate individual scores for GFAP-IR near SCs for each animal.

**4.2.9 Statistical analysis and data presentation**

All statistical analyses were conducted using SPSS 19/20 (IBM; Armonk, NY) and all graphical representations of data were made using GraphPad Prism 5 (GraphPad Software Inc., CA). Images and graphs were processed for presentation using Photoshop CS2 (Adobe). Where the data met parametric assumptions of normality and homogeneity of variance, we conducted independent-samples t-tests to compare the two groups (SKP-SCs and N-SCs). Where the data violated those assumptions, had too small a sample size to judge normality properly (i.e., n<10), or was presented as a percentage, group comparisons were made using the equivalent non-parametric test; namely the Mann-Whitney U test (U). Where appropriate, the degrees of freedom for statistical tests are provided in brackets. The significance level for all tests was set at $p < 0.05$ and 2-tailed test results were reported for all analyses. We examined four batches of each cell type, but given the similarity of results across batches, we treated each well/plate as a separate sample for statistical analyses in assays involving multiple wells/plates per SC batch.
4.3  Results

4.3.1 Cultured SKP-SCs and N-SCs have highly similar phenotypes

We compared the protein and gene expression of SKP-SCs and N-SCs using immunocytochemistry, Western blots and qPCR to analyze the expression of typical markers of SCs at various stages of development. Passage 4 SCs were plated at high density (600-700 cells/mm$^2$) for use in all of these assays to simulate the passage and culture density of SCs typically used in our transplantation experiments. We found similar patterns of immunolabelling for both SKP-SCs and N-SCs using antibodies to p75 (Fig. 4.1A – top panels) and O1 (Fig. 4.1A – bottom panels). p75 is the most commonly used marker for SCs in monoculture because it labels SCs at every developmental stage, except mature myelinating SCs (Jessen and Mirsky, 2005). As such, we used p75 labelling to assess the purity of our SKP-SC and N-SC cultures, and found that purity for all cultures ranged from ~90-95% p75$^+$ cells, and did not differ between the two SC sources ($U_{(14)}=25$, $p=.462$; Fig. 4.1B). O1 (synonymous with GalC) is a marker of mature SCs of both myelinating and non-myelinating phenotypes (Jessen et al., 1987), so we used it here to assess the proportion of mature SCs in SKP-SC and N-SC cultures by counting cells that were positive for both O1 and p75. The percentage of O1$^+$ SCs in each culture ranged from ~10-19%, indicating that most of the SCs in cultures of either SC type had an immature phenotype, and there was no significant difference between the SKP-SCs and N-SCs overall with respect to the percentage of ‘mature’ SCs ($U_{(8)}= 4$, $p=.076$; Fig. 4.1C). In line with the notion that most of the SCs from either source were immature, we found that the vast majority of cells in both SKP-SC and N-SC cultures were positive for GFAP (Fig. 4.1D – left panels), O4 (Fig. 4.1D – middle panels), and S100β (Fig. 4.1D – right panels); markers expressed by SCs from the immature
Figure 4.1  SKP-SCs and N-SCs both label with known SC markers and show similar purity and maturity when grown under identical culture conditions

(A) Sample images of passage 4 SKP-SCs and N-SCs labelled with p75 (green), the nuclear marker Hoechst (Ho, blue), and O1 (red; bottom panels only). (B) Quantification of the percentage of p75\(^+\) SCs ranged from ~90-95\% for all cultures and did not differ significantly between SKP-SCs and N-SCs – indicating high purity of cultures for SCs from either source. (C) Quantification of the percentage of p75\(^+\) SCs that were O1\(^+\) (see arrows in A) also showed no significant difference between SKP-SCs and N-SCs – indicating that SCs from either source showed a similar and relatively low (~10-20\%) proportion of mature cells. (D) Representative images of passage 4 SKP-SCs and N-SCs labelled with nuclear marker (Ho; blue) and GFAP (green; left panels), O4 (red; middle panels), or S100\(\beta\) (red; right panels); note that these typical SC markers labelled nearly all of the cells in cultures from either source of SCs. (B,C) Individual coverslip measures are presented with a solid black line indicating group median values.
stage onwards (Jessen and Mirsky, 2005). These findings indicate that the SKP-SC and N-SC cultures were of equivalent purity and that the majority of cells in cultures of both cell types had an immature SC phenotype.

Western blot and qPCR analyses revealed similar findings with respect to the levels of proteins and mRNA found in lysates collected from passage 4 culture dishes containing 4 million SKP-SCs or N-SCs. Blots of SCs from both sources showed similar levels of all proteins assessed (Fig. 4.2A) and quantification of protein densities from immunoblots for 4 samples of each type of SC indicated no significant differences between SKP-SC and N-SC samples with respect to the levels of NCad ($U_{(6)}=7.00, p=.773$), p75 ($U_{(6)}=6.00, p=.564$), GFAP ($U_{(6)}=6.00, p=.564$), P0 ($U_{(6)}=7.00, p=.773$), or Krox-20 ($U_{(6)}=6.00, p=.564$) proteins (Fig. 4.2B). Gene expression data were normalized to the expression of the GAPDH reference gene and the relative expression of each gene of interest (compared to the average for the N-SC samples) was determined using the $2^{\Delta\Delta C_T}$ method. We compared a minimum of 4 distinct batches of each cell type and found no significant differences between SKP-SCs and N-SCs (Fig. 4.2C) in terms of the expression of the following genes: Cad-19 ($U_{(6)}=6.00, p=.564$), NCad ($U_{(6)}=6.00, p=.564$), p75 ($U_{(6)}=6.00, p=.564$), S100β ($U_{(6)}=8.00, p=1.000$), Id2 ($t_{(13)}=1.082, p=.299$), Id4 ($U_{(6)}=5.00, p=.386$), Pax3 ($t_{(9,867)}=1.48, p=.170$), GalC ($U_{(6)}=6.00, p=.564$), P0 ($U_{(6)}=6.00, p=.564$), Krox-20 ($t_{(13)}=.877, p=.396$), MBP ($U_{(6)}=4.00, p=.248$), or Cdc2 ($U_{(6)}=7.00, p=.773$). These findings demonstrate that under the conditions we use to expand cells for transplantation, SKP-SCs and N-SCs express similar levels of a variety of proteins and genes associated with various stages of SC development.
Figure 4.2 SKP-SCs and N-SCs show similar levels of expression for a variety of proteins and genes related to SC development in vitro

(A) Representative immunoblots for proteins involved in various stages of SC development and associated loading controls run on passage 4 SKP-SC and N-SC samples. (B) Quantification of Western blot results depicting the average optical density of each protein relative to loading controls (Actin or GAPDH as indicated in A). (C) Graphical depiction of qPCR results comparing the relative expression of genes (normalized to the GAPDH reference gene) involved in various stages of SC development. Note that the relative expression of all genes of interest and the relative amounts of all proteins measured were similar for SKP-SCs and N-SCs – indicating substantial overlap in the expression patterns of markers typically associated with SCs at various stages of development in SCs from both sources. All data are presented as the mean (±SEM) from 4-8 samples per group.
4.3.2 SKP-SCs and N-SCs both show poor migration over astrocytes in vitro

The inverted coverslip migration assay has been used extensively to demonstrate the limited migration of N-SCs over astrocyte monolayers, as well as to study the underlying mechanisms of this phenomenon and test potential treatments designed to promote SC migration in astrocytic environments (Wilby et al., 1999; Lakatos et al., 2000; Afshari et al., 2010a; Afshari et al., 2010b). Here we used that assay to compare the migratory potential of SCs generated from SKPs and nerve. Of the 50,000 SKP-SCs or N-SCs initially plated on each coverslip fragment, only a handful of each type of SC had migrated out from any one side of a coverslip on average after 2 days (Fig. 4.3A). Only healthy, CT dye-labelled, p75+ cells were included in counts (see Fig. 4.3B for ideal examples). The vast majority of those cells were found within 100 µm of the coverslip edge and there were no significant differences between the average number of SKP-SCs and N-SCs that migrated 0-0.1mm ($U_{(27)}$=56.50, $p$=.055), 0.1-0.5mm ($U_{(27)}$=96.50, $p$=.894), 0.5-1mm ($U_{(27)}$=87.00, $p$=.509), or 1-2mm ($U_{(27)}$=66.00, $p$=.070) over astrocyte monolayers (Fig. 4.3C). These results indicate that neonatal rat SKP-SCs and N-SCs show similar migratory potential on astrocyte monolayers in vitro, which implies that astrocytes are just as inhibitory to SKP-SC migration as they are to N-SC migration. In support of that notion, we found that the numbers of migrating SKP-SCs and N-SCs observed here were similar to multiple results reported previously for N-SCs on astrocytes in the same assay, and much lower than the number of migrating SCs typically seen on permissive substrates, such as laminin (Wilby et al., 1999; Lakatos et al., 2000; Afshari et al., 2010a; Afshari et al., 2010b).
Figure 4.3 SKP-SCs and N-SCs both show poor migratory potential on astrocytes in vitro

(A,B) Sample images of SKP-SCs and N-SCs labelled with cell tracker dye (green) and p75 (red), as well as the nuclear marker Hoechst (blue), as they migrate out from under a coverslip and on to an astrocyte monolayer in the inverted coverslip migration assay. Cells were counted as migrating SCs if they were found outside of the area covered by the inverted coverslip and showed labelling for both cell tracker dye and p75 – as depicted in (B). (C) Quantification of the migration of SKP-SCs and N-SCs over astrocytes at various distances from the coverslip; data presented as group mean ± SEM. Note that both SKP-SCs and N-SCs migrate poorly over CNS astrocyte monolayers in vitro and there is no significant advantage for SCs from either source.
4.3.3 SKP-SCs and N-SCs exhibit similar boundary formation with astrocytes in vitro

The SC-astrocyte boundary assay provides a model for studying SC-astrocyte interactions similar to those that occur naturally at the glial limitans or following the transplantation of N-SCs into the injured spinal cord. Previous studies have demonstrated that N-SCs show limited integration with astrocytes in this assay, and a number of works have linked that finding to enhanced astrocyte reactivity, as indicated by hypertrophy and increased expression of GFAP and inhibitory proteoglycans, in response to confrontation with N-SCs (Wilby et al., 1999; Lakatos et al., 2000; Fairless et al., 2005; Afshari et al., 2010a). Similar to those results, at one month after plating, we found that a distinct border had formed between the SCs and astrocytes in all wells containing either SKP-SCs or N-SCs (Fig. 4.4A). Small patches of SCs were observed on the astrocyte side of the border (arrows in Fig. 4.4A), but these SCs were largely found occupying small spaces between the astrocytes near the border, rather than migrating over the top of those cells. Where SCs did extend into the astrocyte area, the average area they occupied did not differ significantly between the SKP-SCs and N-SCs ($t_{(27)}=0.53, p=0.958$; Fig. 4.4B). In addition, the reactivity of astrocytes in confrontation with SKP-SCs and N-SCs appeared similar, as there was no significant difference between the average GFAP-IR of astrocytes cultured with SKP-SCs versus those cultured with N-SCs ($t_{(26)}=-0.159, p=0.875$; Fig. 4.4C). These results indicate that SKP-SCs and N-SCs exhibit similar boundary formation with astrocytes, and that after one month in vitro SCs from either source elicit similar levels of astrocyte reactivity.
Figure 4.4 SKP-SCs and N-SCs show similar interactions with astrocytes in vitro in SC-astrocyte boundary assays

(A) Representative images of SKP-SCs (top) and N-SCs (bottom) labelled with p75 (green) in boundary assays with CNS astrocytes labelled with GFAP (red). Note the formation of distinct boundaries between the SCs and astrocytes in both SKP-SC and N-SC samples. Arrowheads point at regions of overlap between SCs and astrocytes on the astrocyte side of the boundary – an indication of SC migration into astrocyte-rich regions. (B) Quantification of SC integration in astrocyte-rich regions based on the area of p75+ and GFAP+ overlap on the astrocyte side of the cellular boundary normalized to the length of the border of SC-astrocyte contact in each well. Average area of SC-astrocyte overlap did not differ significantly between SKP-SCs and N-SCs. (C) Quantification of GFAP+ pixel intensity on the astrocyte side of the SC-astrocyte boundary normalized to the GFAP+ pixel intensity on the SC side of that boundary. The lack of difference between SKP-SCs and N-SCs on this measure indicates similar levels of astrocyte reactivity in opposition to SCs from either source. All data are presented as group mean ± SEM.
4.3.4 SKP-SCs and N-SCs display similar integration and migration potentials following transplantation into the injured rodent spinal cord

To compare the integration and migration potentials of SKP-SCs and N-SCs in vivo we transplanted GFP$^+$ cells directly into the lesion site immediately following an incomplete cervical SCI. By 11 weeks post-injury nearly all of the surviving SCs from both sources had migrated out of the lesion cavity and into spared, if not completely intact, host parenchyma (Fig. 4.5A,B). The majority of these cells were found in the spared rim and mid-lesion bridges, where GFAP$^+$ cells are present, but display an abnormal cytoarchitecture indicative of ‘lesioned’ tissue. However, some SCs from both sources were found in relatively intact GFAP$^+$ host parenchyma as well (arrowheads in Fig. 4.5A), and occasionally transplant-derived cells were observed migrating further out from the lesion site in relatively normal host tissue (arrows in Fig. 4.5B). Quantification of the area occupied by SCs in those regions (i.e., outside of the lesion), demonstrated no significant difference between SKP-SCs and N-SCs in terms of their integration with host astrocytes ($U_{(20)}=44.00, p=.291$; Fig. 4.5C), and the maximum distance of SC migration from the lesion site also showed no significant difference between SKP-SCs and N-SCs in terms of their ability to migrate through astrocyte-rich host parenchyma ($U_{(20)}=52.00, p=.597$; Fig. 4.5D). In addition, an analysis for GFAP-immunoreactivity in regions of host tissue adjacent to transplant-derived cells indicated no significant difference in the level of GFAP produced by astrocytes in the vicinity of SCs from either source ($t_{(20)}=-1.714, p=.102$; Fig. 4.5E). These results indicate that SKP-SCs and N-SCs from neonatal sources migrate and integrate with astrocyte-rich host tissue to a similar extent following transplantation into the injured rodent CNS, and that host astrocytes show similar levels of reactivity in the presence of SCs from either source.
Figure 4.5 SKP-SCs and N-SCs show similar integration and migration potential in the injured cervical spinal cord

(A,B) Sample images of GFP\(^+\) (green) SKP-SCs (top panels) and N-SCs (bottom panels) that have migrated out of the lesion cavity and into GFAP\(^+\) (blue) host parenchyma 11 weeks after transplantation into the injured cervical spinal cord. These cells were largely found immediately adjacent to the lesion cavity (* in A,B), around the edge of the injury site, or in the spared rim and mid-lesion bridges, where the GFAP\(^+\) astrocytes display abnormal cytoarchitecture. However, SCs from both sources were occasionally observed in GFAP\(^+\) regions with relatively normal cytoarchitecture (arrowheads in A) and small patches of those cells were also observed further out from the lesion site in relatively intact tissue (arrows in B). (C-E) Quantifications of area occupied by GFP\(^+\) SCs outside of the lesion (C), maximum GFP\(^+\) SC migration from the lesion (D), and GFAP-immunoreactivity near transplant-derived SCs (E) all failed to find significant differences between transplanted SKP-SCs and N-SCs. Individual data points for each animal are presented with group medians indicated by solid black lines in C and D. Data are presented as mean (± SEM) in E.
4.4 Discussion

Towards establishing the suitability of SKP-SCs as an alternative to N-SCs for therapeutic application in SCI, herein we examined the purported phenotypic and functional differences between the SCs generated from those two sources by conducting a variety of comparisons between SKP-SCs and N-SCs generated from neonatal rat tissue and cultured under conditions similar to those we typically use to generate cells for our preclinical transplantation experiments. Based on findings from our previous studies (Chapter 2; Assinck et al., submitted) we hypothesized that the SKP-SCs possess a less mature SC phenotype than N-SCs, and we predicted that the SKP-SCs would induce less reactivity from local astrocytes and display enhanced migration/integration into astrocyte-rich territory compared to N-SCs both in vitro and in vivo. However, contrary to those hypotheses, SKP-SCs and N-SCs did not differ significantly on any of the assays we conducted, suggesting that SCs generated from neonatal SKPs possess a phenotype that is highly similar to that of SCs harvested from neonatal peripheral nerve, particularly when those cells are expanded in culture under identical conditions over multiple passages.

SKP-SCs have previously been shown to express a variety of characteristic SC marker proteins, including: p75, GFAP, S100β, P₀, peripheral myelin protein 22 kDa and MBP (Biernaskie et al., 2006; McKenzie et al., 2006; Fernandes et al., 2008), and here we demonstrated for the first time that neonatal SKP-SCs also produce O1, O4, NCad and Krox-20, and express the following genes: Cad-19, Id2, Id4, Pax3, and Cdc2. Although some of those molecules and genes are known to be involved in SC development at multiple stages (p75, S100β, and P₀), others are limited to association with specific stages of SC development or
differentiation, such as: Cad-19 and NCad (expressed by SC precursors); Pax3 and Id2/4 (expressed by immature SCs); O1/GalC (expressed by mature SCs); Krox-20 and MBP (expressed by mature pro-myelinating and myelinating SCs); and Cdc2 (expressed by dedifferentiated SCs following nerve injury) (Jessen and Mirsky, 2005; Han et al., 2007). Here we found that neonatal SKP-SCs express all of those molecules/genes, and where quantified, the levels of expression of those factors were equivalent in cultured neonatal SKP-SCs and N-SCs grown under the same conditions. Thus, our limited examination of the expression profiles of those cells produced no evidence to support the notion that neonatal SKP-SCs possess a less mature SC phenotype than their nerve-derived counterparts. Furthermore, given that cultures of both neonatal SKP-SCs and N-SCs expressed all of those markers to some degree, our findings indicate that SCs generated from either of those sources contain a mixture of cells at various stages of differentiation/maturation, and the low proportion of cells that labelled with the mature SC marker O1 (ranging from ~10-20%) across cultures suggests that cultured neonatal SKP-SCs and N-SCs both primarily possess an immature SC phenotype.

Compared to the transplantation of N-SCs generated from adult rodent nerve (e.g., Pearse et al., 2004b; Barakat et al., 2005), our previous results transplanting neonatal/adult rodent SKP-SCs into thoracic contusion sites in the rat suggested that the SKP-SCs induce less reactivity from host astrocytes, migrate further into intact astrocyte-rich host parenchyma, integrate better with astrocytes at the edge of the lesion, and support greater axonal growth, particularly across the distal graft-host interface (Chapter 2; Assinck et al., submitted). However, such comparisons between independent studies conducted by different laboratories may be confounded by many uncontrolled variables; hence we conducted a direct side-by-side assessment of SCs from those
two sources here. Contrary to hypotheses based on our previous work, the present results demonstrated that SKP-SCs are highly similar to N-SCs in terms of the degree of reactive astrogliosis they elicit in nearby astrocytes and their ability to migrate/integrate into astrocyte-rich territory both *in vitro* and *in vivo* when both of those cell types are generated from neonatal tissue sources. Concurrent with the present work, we conducted a study examining the efficacy of neonatal SKP-SCs and N-SCs transplanted into the partially crushed cervical spinal cord in rats. That study found only limited evidence that neonatal SKP-SCs may induce less reactive astrogliosis (i.e., less GFAP expression in host tissue immediately rostral to the lesion site) and promote greater supraspinal axon growth/sparing (i.e., enhanced rubrospinal axon density in the gray matter rostral to the lesion site) than their nerve-derived counterparts, and showed that both types of SCs support similar levels of growth/sparing of putative brainstem-spinal serotonergic and noradrenergic axons (Chapter 3). Taken together those findings suggest that neonatal N-SCs may be more similar to SKP-SCs than adult N-SCs are, particularly in terms of their abilities to migrate/integrate into astrocyte-rich domains and promote the growth/sparing of certain axon populations at the level of the lesion. Thus, the results of our SKP-SC transplantation work to date suggest that SKP-SCs (whether neonatal or adult) may display more robust advantages over adult N-SCs than they do over neonatal N-SCs following transplantation into the injured CNS.

SKP-SCs are generally thought to be less mature than N-SCs because, unlike the latter, the former cells are generated directly from precursors in culture in the absence of axons and have therefore never differentiated into mature myelinating or non-myelinating SCs. However, given that the neonatal N-SCs examined here were harvested from peripheral nerve at postnatal day 1-2, prior to the completion of radial sorting and the conversion of immature SCs to mature.
myelinating and non-myelinating SC phenotypes (Webster and Favilla, 1984; Woodhoo and Sommer, 2008), that population of cells may include a substantial proportion of SCs that were still at the immature stage of SC development at the time of isolation. Thus, the neonatal N-SCs examined in the present work may represent a population of cells that is more akin to immature SCs than N-SCs isolated from adult tissue, and if that is the case, then the similarity between neonatal N-SCs and SKP-SCs would imply that both of those cell types may represent a less mature population of SCs than adult N-SCs.

The notion that neonatal N-SCs possess a phenotype that is more similar to that of SKP-SCs than N-SCs generated from adult nerve, implies that neonatal N-SCs should also have advantages over adult N-SCs, similar to those displayed by the SKP-SCs. The results of the in vivo assays in the present work provide some support for that hypothesis, as neonatal N-SCs appeared to elicit relatively little reactivity in nearby astrocytes and migrated/integrated into astrocyte-rich spared host tissue more extensively than one would expect based on previous work with N-SCs from adult tissue sources. The same cannot be said of those cells in the SC-astrocyte co-culture models used here, but both SKP-SCs and N-SCs appeared to elicit relatively high levels of reactive astrogliosis and showed quite poor migration and integration into astrocyte-rich regions in vitro; which suggests that the co-culture assays used here may provide an environment that is even less favourable to SC migration/integration than the injured spinal cord. Those results may be due to our use of the shaker method to purify astrocytes in the present work, as that method was recently shown to produce astrocytes with a more reactive phenotype than other methods of astrocyte isolation/purification (Zamanian et al., 2012). Alternatively, it may be the case that the SCs interact differently with host tissue due to presence of other cell types (e.g.,
neurons) or signals (e.g., denuded/damaged axons) in vivo that are lacking from the in vitro environment. Although the notion that neonatal and adult N-SCs differ in terms of their interactions with astrocytes may seem counterintuitive because both of those cell types have been reported to elicit reactive astrogliosis and show relatively poor migration/integration into astrocyte-rich territories (Lakatos et al., 2000; Grimpe et al., 2005; Andrews and Stelzner, 2007; Pearse et al., 2007; Santos-Silva et al., 2007; Schaal et al., 2007), to our knowledge N-SCs from neonatal and adult tissue sources have never been directly compared to one another with respect to those parameters in a single experiment either in vitro or in vivo. Thus, subtle differences between those two cell types may exist that have simply not been recognized or characterized to date; which represents another potential issue for future investigation.

One potentially important limitation to the work we have conducted to date directly comparing the interactions of SKP-SCs and N-SCs with astrocytes in vitro and in vivo is the limited number of markers of astrocyte reactivity that have been utilized. Reactive astrogliosis is a heterogeneous state that varies widely depending on the type of insult inflicted on the CNS. Indeed, using gene arrays, Zamanian et al. (2012) found that ~50% of the changes in astrocyte gene expression following middle cerebral artery occlusion and lipopolysaccharide injections differ; indicating that the phenotype of reactive astrocytes is highly injury specific. The characterization of reactive astrocyte phenotypes following SCI and in the presence of SCs has yet to be examined so comprehensively, and should be a priority in the future. Along the same lines, future work comparing SKP-SCs and N-SCs should include a wider array of measures of reactive astrogliosis to ensure that differences between those two cell types are not being overlooked. A more comprehensive comparison of the interactions between host astrocytes and
transplanted cells may include well established measures previously applied to N-SCs alone (e.g., astrocyte hypertrophy and the expression of CSPGs other than Neurocan), as well as novel markers suggested by more recent work (e.g., expression of serpina3n, pentraxin 3, and transglutaminase 1) (Zamanian et al., 2012).

In summary, the present work demonstrated that neonatal rat SKP-SCs and N-SCs expanded over multiple passages in vitro (as for transplantation), have mixed, but primarily immature, SC phenotypes and are indistinguishable in terms of their expression of many SC developmental markers and their interactions with astrocytes both in vitro and in vivo. The high degree of similarity between SKP-SCs and N-SCs found in the present work contributes to a growing body of evidence suggesting that SKP-SCs are a suitable alternative to N-SCs for therapeutic application after SCI. Combined with our previous findings regarding the similarities and differences between SKP-SCs and N-SCs (Chapter 2; Chapter 3; Assinck et al., submitted), our results here also suggest that SKP-SCs in general (i.e., regardless of the age of their source tissue) may be more similar to neonatal N-SCs than they are to adult N-SCs; which is in line with the idea that the SKP-SCs may represent a less mature SC phenotype than their counterparts generated from adult peripheral nerve. That notion should be addressed in future work by comparing SKP-SCs generated from adult skin to N-SCs harvested from embryonic, neonatal and adult tissue sources, to determine whether adult SKP-SCs do indeed represent a less mature SC phenotype than their clinically relevant adult nerve-derived counterparts.
Chapter 5:

General Discussion
5.1 Opening statement

SC transplantation is one potential therapy that continues to show promise as a clinical treatment for SCI, as SCs harvested from peripheral nerve and expanded in vitro are well known to promote repair and functional recovery in animal models of SCI (Bunge and Wood, 2012) and preliminary clinical assessments of N-SC transplantation in humans with SCI have demonstrated safety and some signs of efficacy (Saberi et al., 2008; Saberi et al., 2011). However, the generation of those cells from autologous sources causes permanent injury to the PNS. In humans, the sural nerve is typically excised to generate N-SCs for the purposes of clinical application, and the resulting injury to that nerve is associated with persistent sensory deficits and the risk of developing abnormal sensations including persistent neuropathic pain. One way to avoid those complications is to generate SCs from autologous sources other than peripheral nerve. SKPs represent one such alternative source for the generation of autologous SCs, as the harvest of skin is less invasive than the excision of peripheral nerve and carries no risk of permanent functional loss or neuroma formation. This thesis focused on examining the therapeutic potential of SKP-SCs as a treatment for SCI and comparing those cells to N-SCs to determine whether SCs generated from precursors in mammalian skin are a suitable alternative those harvested from peripheral nerve for transplantation-based CNS repair.

5.2 Summary of thesis

Chapter 2 describes our initial work examining the efficacy of naïve SKPs and SKP-SCs as therapeutic candidates for SCI using the highly clinically relevant thoracic contusion model (Biernaskie et al., 2007). In this work, SKP-SCs, naïve SKPs, and neurospheres (cellular control
treatment) were generated from YFP+ neonatal mouse tissue and transplanted one week after a moderate thoracic contusion. At 12 weeks post-injury, rats receiving SKP-SCs showed improvement on a variety of histolopathological and behavioral outcomes compared to those receiving naïve SKPs or neurospheres, thus demonstrating the efficacy of SKP-SCs as a treatment for SCI, and particularly thoracic contusion injuries. Although transplanted naïve SKPs filled the lesion site and differentiated into myelinating SCs in that region, they also differentiated into undesirable mesodermal cell types, and treatment with naïve SKPs was associated with enhanced sensitivity to sensory stimuli (similar to previous work with undifferentiated CNS neural stem cells; Hofstetter et al., 2005) and no significant improvement in locomotor recovery compared to control treatments. In contrast, SKP-SCs only labelled with SC markers and displayed typical SC morphologies (i.e., bipolar p75+ non-myelinating or tubular P0+ myelinating phenotypes), and those cells appeared to be ideally suited to therapeutic transplantation after SCI, as they bridged the lesion cavity, enhanced tissue sparing, supported axonal growth into and even through the lesion site, myelinated host axons within the lesion site and the spared rim, and induced extensive recruitment of endogenous SCs that contributed substantially to remyelination in the injured CNS. In agreement with the histological evidence, rats receiving SKP-SCs showed improved hindlimb locomotor function relative to other treatment groups and no indication of lowered sensory thresholds. Thus the findings in Chapter 2 of this thesis provided the first demonstration of efficacy for SKP-SCs as a treatment for SCI and clearly demonstrated that naïve SKPs are not suitable for transplantation-based repair of the injured CNS.
In comparing our results transplanting neonatal mouse SKP-SCs to previous reports by other groups transplanting adult N-SCs under similar conditions (e.g., Pearse et al., 2004), we noted that although the SKP-SCs shared many of the behavioural properties and reparative benefits commonly attributed to N-SCs transplanted into the injured spinal cord, they also appeared to differ from those nerve-derived cells in ways that may be advantageous with respect to their suitability and capacity for CNS repair. For example, unlike transplanted N-SCs, SKP-SCs did not form distinct boundaries with astrocytes. In fact, the SKP-SCs intermingled with astrocytes that invaded the graft, and were often found to extend from the rostral and caudal borders of the graft into astrocyte-rich host tissue. In addition, although the SKP-SCs largely remained within the graft/lesion, they were occasionally found to migrate a considerable distance within the intact parenchyma, and could escape the graft to myelinate denuded axons within the adjacent rim of spared tissue. Furthermore, SKP-SCs did not elicit elevated GFAP expression in areas where they integrated with astrocytes, and actually appeared to elicit lower expression of the CSPG neurocan than naïve SKPs and neurospheres. Finally, unlike N-SCs transplanted into sub-acute thoracic contusions, SKP-SCs alone (i.e., in the absence of additional co-treatments) promoted the growth of descending serotonergic and noradrenergic fibres (putative brainstem-spinal axons) and appeared to support the growth of those and other fibres (e.g., ascending sensory axons) across the caudal and rostral graft-host interfaces, respectively. Thus, compared to previous reports regarding N-SCs, our findings in Chapter 2 suggested that the SKP-SCs have advantages over N-SCs in terms of their interactions with astrocyte-rich spared host tissue and their ability to support axonal growth at the level of the lesion in the contused thoracic spinal cord of the rat.
Based on that evidence, we hypothesized that SKP-SCs, although highly similar to N-SCs, differ from their nerve-derived counterparts in a manner that enables the SKP-SCs to migrate and integrate into astrocyte-rich host tissue more extensively and support more robust axonal growth from supraspinal axon populations, particularly across the distal graft-host interface. Furthermore we speculated that the SKP-SCs possess those properties because they represent a less mature SC phenotype than dedifferentiated N-SCs; a notion supported by the fact that SCPs harvested from embryonic nerve are known to display similar attributes when transplanted into the demyelinated/injured CNS (Woodhoo et al., 2007; Agudo et al., 2008). Lastly, we hypothesized that the improved migration and integration of SKP-SCs with host tissue and their enhanced capacity to promote axonal growth in the injured CNS would make the SKP-SCs more effective than N-SCs with respect to CNS repair, and as such we predicted that SKP-SCs would convey greater functional benefit than N-SCs when applied to the same injury model.

To test those hypotheses, we conducted two additional overlapping studies, described here in Chapter 3 and 4. Given that neonatal N-SCs are typically characterized as having the same limitations (i.e., with respect to their interactions with astrocytes, migration/integration into astrocyte-rich territory, and ability to support axonal growth at the level of the lesion) as adult N-SCs (Keirstead et al., 1999a; Lakatos et al., 2000; Plant et al., 2001; Lakatos et al., 2003; Azanchi et al., 2004; Andrews and Stelzner, 2007; Pearse et al., 2007), we chose to focus our comparison of SKP-SCs and N-SCs on cells generated from neonatal rat tissue. Furthermore, in light of the fact that our interest in these cells primarily relates to their potential use as a treatment for SCI, all of our comparisons between SKP-SCs and N-SCs were conducted using
cells expanded in vitro to passage 3/4 using methods similar to those we typically use to generate cells for transplantation in our pre-clinical SCI studies.

The work described in Chapter 3, examined the efficacy of SKP-SCs as a treatment for incomplete cervical SCI, and included two experiments; the first comparing neonatal SKP-SCs versus media treatment and the second comparing neonatal SKP-SC, N-SC or dermal fibroblast transplants. The goal was to establish the SKP-SCs as a potential treatment for cervical injury, to examine the ability of those cells to promote supraspinal axon growth (specifically of the RST), and to directly compare SKP-SCs and N-SCs transplanted into the injured CNS for the first time. Both experiments involved acute treatment of a left DLF crush injury at the cervical level with an 11 week post-injury/treatment endpoint. In order to examine the effects of immunosuppression on the outcome of SKP-SC transplantation, half of the animals in each treatment group were administered CsA for the duration of the first experiment. In contrast, all of the animals were given CsA throughout the second experiment. Despite the fact that graft survival appeared to be compromised in all of the transplantation groups (likely due to the use of acute transplantation), significant behavioural, electrophysiological and histopathological differences were found among the groups, and generally indicated the efficacy of SKP-SC and/or N-SC transplantation therapies for incomplete cervical SCI.

Compared to media treatment, SKP-SC transplantation was associated with increased use of the forelimb affected by injury (cylinder test), improved RST electrophysiological functions (relative to uninjured controls), a similar level of reactive astrogliosis, and increased sparing/plasticity of the rubrospinal axon branches in the gray matter rostral and caudal to injury. Curiously, the latter effect only reached significance when comparing SKP-SC and media treated
animals that had not received CsA during the study, as CsA treatment tended to increase RST axon densities in the media-treated group and decrease them in the SKP-SC-treated group, but appeared to have no significant effect on any other outcome measure assessed in the study. Thus the findings from Experiment 1 in Chapter 3 demonstrated the efficacy of SKP-SCs as a treatment for incomplete cervical crush SCI and found evidence to suggest that immunosuppression with CsA may reduce the efficacy of those cells by limiting their ability to support the growth/sparing of certain axon populations.

Compared to fibroblast treatment, transplantation of either type of SC was associated with the increased preservation of host tissue within and adjacent to the lesion site, improved distribution of weight to the injured forelimb during locomotion (Catwalk) and reduced thresholds of stimulation for EMG responses in the distal forelimb musculature. In addition, the SKP-SC group alone demonstrated lower EMG latencies (relative to uninjured controls), reduced RST atrophy and increased sparing/plasticity of RST axon branches in the gray matter rostral to injury. Thus Experiment 2 of Chapter 3 demonstrated that the transplantation of either SKP-SCs or N-SCs is preferable to that of fibroblasts for treating SCI, particularly in the case of partial crush injuries at the cervical level.

Following acute transplantation into the partially injured cervical spinal cord, neonatal SKP-SCs and N-SCs demonstrated highly comparable behaviour and reparative effects. Similar to the results of previous work transplanting SKP-SCs and N-SCs, surviving SCs from both sources generated myelinating (P0+) and non-myelinating (p75+) SCs in the injured CNS, promoted the preservation of the spared tissue rim and mid-lesion bridges, enhanced the endogenous SC response, and supported axonal growth/sparing in regions occupied by grafted
cells (i.e., the spared rim and mid-lesion bridges). As expected, there were no significant differences between the outcomes of transplanting SCs generated from SKPs versus nerve in terms of graft survival, neuroprotection (lesion/cavity volume and spared rim width), myelination potential (GFP⁺/P₀⁺ SC volume), or endogenous SC recruitment (P₀⁺/GFP⁻ SC volume). However, contrary to our hypotheses, neither of those cell types prevented the dieback of injured RST axons nor prompted any direct regeneration of that tract in the intact white matter, and the SKP-SCs and N-SCs appeared to support a similar degree of axonal growth/sparing at the level of the lesion, including SERT- and TH-positive (putative brainstem-spinal) axon populations. In addition, although transplanted N-SCs were found to elicit higher GFAP expression than SKP-SCs, that effect was only observed in spared tissue immediately rostral to the injury site and was not associated with enhanced CSPG production in that region.

Although SCs from both sources induced similar levels of axon growth at the lesion site, the SKP-SCs were found to significantly enhance the sparing/plasticity of the RST branches in the gray matter rostral to the lesion site compared to N-SCs. With respect to functional recovery, the only difference noted between those two cell types was that the SKP-SCs appeared to promote slightly better electrophysiological recovery, as unlike the N-SC group, the SKP-SC group demonstrated distal forelimb EMG latencies that did not differ significantly from uninjured controls. However, it is important to note that there were no direct statistically significant differences between the two SC groups in terms of either electrophysiological or behavioural outcomes. Thus, although neonatal SKP-SCs promoted greater RST sparing/plasticity in the gray matter and less GFAP-expression by astrocytes rostral to injury, those discrete neuroreparative advantages generally failed to translate into a meaningful
difference in functional recovery compared to neonatal N-SCs. As such, our work in Chapter 3
transplanting neonatal SKP-SCs and N-SCs largely failed to find the anticipated differences
between those cell types.

Chapter 4 focused on directly comparing cultured SKP-SCs and N-SCs with respect to
their SC phenotype *in vitro* and their interactions with astrocytes both *in vitro* and *in vivo*. The
goal of this work was to determine whether SKP-SCs indeed represent a less mature SC
phenotype than dedifferentiated N-SCs and whether SCs from those two sources differ in terms
of their ability to migrate and integrate into astrocyte-rich domains. To address these questions I
compared the expression of a variety of characteristic SC markers in cultures of SKP-SCs and N-
SCs to determine whether SKP-SCs express higher levels of proteins/genes associated with less
mature stages of SC development. I also examined the astrocyte response to SCs from both
sources, as well as the ability of those cells to migrate and integrate into astrocyte-rich territory
both *in vitro* and *in vivo*. Contrary to the hypotheses based on our previous work, I found no
significant differences between SKP-SCs and N-SCs on any of these parameters, when both cell
types were isolated from neonatal rat tissue, purified and expanded in culture under conditions
similar to those typically used to generate cells for transplantation.

In particular, these analyses revealed that cultured SKP-SCs and N-SCs represent a
mixed population of cells that express markers of various stages of SC development, but were
both primarily comprised of cells with relatively immature SC phenotypes. As a population of
cells, the SKP-SCs and N-SCs did not differ significantly in terms of the expression of any of the
proteins/genes associated with SC development that I tested, which suggests that SCs from those
two sources generally share similar SC phenotypes in terms of their differentiation/maturation.
state. *In vitro* SCs from either source exhibited comparably poor migration over astrocyte monolayers and formed distinct boundaries with astrocytes in the confrontation assay, displaying little integration and eliciting similar levels of astrocyte reactivity. However, at 11 weeks after transplantation into the injured cervical spinal cord, the surviving transplant-derived SCs from both sources were primarily found in the spared rim of tissue at the edge of the spinal cord and the mid-lesion bridges, and although the cells tended to occupy regions of the parenchyma with abnormal astrocytic cytoarchitecture, a considerable portion of cells from either source were found in regions of relatively intact astrocyte-rich parenchyma. Also, a small number of SKP-SCs and N-SCs were found further away from the lesion in astrocyte-rich host tissue that showed no signs of injury. Furthermore, both SKP-SCs and N-SCs appeared to integrate well with spared host tissue, eliciting little (and comparable levels of) reactivity in nearby astrocytes.

The results indicate that cultured SKP-SCs and N-SCs generated from neonatal rat tissue were both able to migrate/integrate into astrocyte-rich spared host tissue while eliciting little reactive astrogliosis *in vivo*, whereas neither cell type appeared able to mimic that behaviour *in vitro*. This was interpreted as evidence that the co-culture assays may provide an environment that is even less conducive to SC migration than the injured spinal cord. Regardless of the apparent differences between those models, neonatal SKP-SCs and N-SCs did not differ from one another in terms of their astrocyte interactions in either case, and these cells were also found to share highly similar SC phenotypes according to their protein/gene expression profiles. Thus my work in Chapter 4 largely confirmed the high degree of similarity between neonatal rat SKP-SCs and N-SCs indicated in our previous direct comparison of those cells (Chapter 3), and much like that work, the findings in Chapter 4 also contradicted the hypotheses we made based on
comparisons between our previous work transplanting neonatal SKP-SCs (Chapter 2) and the work of others transplanting N-SCs under similar conditions.

5.3 Summary of comparisons between neonatal SKP-SCs and adult/neonatal N-SCs

In Chapter 2 we found evidence to suggest that neonatal SKP-SCs have advantages over adult N-SCs with respect to the degree of reactive astrogliosis they induce and their abilities to migrate and integrate into astrocyte-rich domains and support axonal growth at the level of the lesion. Based on the assumption that neonatal and adult N-SCs share similar properties in terms of their interactions with astrocytes and their abilities to promote axonal growth in the injured spinal cord, we predicted that neonatal SKP-SCs would show the same advantages over neonatal N-SCs in our follow-up studies described in Chapter 3 and 4. However, when we directly compared SKP-SCs and N-SCs generated from neonatal rat tissue on a variety of in vitro assays and following transplantation into the partially injured cervical spinal cord, our results largely failed to confirm the anticipated differences between those cell types, and instead indicated that those cell types shared highly similar phenotypes in terms of their expression of proteins/genes associated with SC development, their ability to migrate/integrate into astrocyte-rich domains, and their capacity to support axonal growth at the level of the lesion. Thus it would appear that neonatal SKP-SCs have advantages over adult N-SCs that they do not have over neonatal N-SCs.

Indeed, the only significant differences we found between SKP-SCs and N-SCs generated from neonatal tissue were that the SKP-SCs induced less GFAP expression in host tissue immediately rostral to the injury site and enhanced sparing/plasticity of the RST branches in the gray matter rostral to injury following DLF crush at the cervical level. The functional relevance
of these effects remain questionable because although the SKP-SCs had distal forelimb EMG latencies closer to those of uninjured controls, the SKP-SC and N-SC groups were not significantly different from one another on any electrophysiological or behavioural measures. With respect to axonal growth, similar results were reported by Midha and colleagues, who found evidence to indicate that neonatal SKP-SCs promote enhanced regeneration of PNS axons and improved electrophysiological outcomes compared to neonatal N-SCs following transplantation into an acellular (freeze-thawed) nerve graft or into the chronically denervated nerve itself following direct repair (Walsh et al., 2009; Walsh et al., 2010). In addition, those authors demonstrated that neonatal SKP-SCs produced significantly higher levels of NGF and NT-3 than their age-matched nerve-derived counterparts in culture; suggesting a likely mechanism for their superior in vivo effects (Walsh et al., 2009). A similar mechanism is likely to underlie the differential RST response to neonatal SKP-SCs and N-SCs observed in the present work (Chapter 3), particularly given the distance between the transplanted cells and the RST fibres they appeared to be influencing.

Thus, although neonatal SKP-SCs and N-SCs appear to share largely similar phenotypes, it seems that neonatal SKP-SCs do have some advantages over neonatal N-SCs. However, to date the advantages appear to be limited to the induction of less astrocyte reactivity, the increased production of some neurotrophins, and potentially related improvements in the growth/sparing of certain axon populations and/or the recovery of electrophysiological functions.
5.4 Do neonatal N-SCs have advantages over adult N-SCs?

The notion that neonatal SKP-SCs are more similar to neonatal N-SCs than they are to adult N-SCs carries another important implication, as it obviously suggests that neonatal N-SCs should have advantages over adult N-SCs that are similar to those displayed by SKP-SCs. Indeed, our results in Chapter 4 support that notion, as the neonatal N-SCs transplanted into a DLF crush at the cervical level were found to migrate out of the lesion site and integrate with astrocyte-rich host tissue without eliciting much reactive astrogliosis in the process. The idea that SCs harvested from postnatal nerves at different ages may differ in terms of their suitability for CNS applications is rather novel, as such advantages have only really been suggested previously for SCPs (Woodhoo et al., 2007; Agudo et al., 2008). However, it is important to note that N-SCs harvested from neonatal and adult tissues have previously been shown to differ in some ways, as those cells have long been known to display differential proliferation responses to SC mitogens in culture (Dong et al., 1997).

Intuitively it makes sense that neonatal N-SCs would differ from adult N-SCs, as the population of N-SCs harvested from neonatal nerve is likely made up of a mixture of immature SCs and SCs that had yet to fully differentiate into mature myelinating and non-myelinating phenotypes. As such, as a population, the neonatal N-SCs probably have to undergo less dedifferentiation to return to an immature/proliferative SC state. In contrast, cultures of adult N-SCs, which are harvested well after the completion of development, are comprised entirely of cells that have to dedifferentiate from fully mature myelinating and non-myelinating SCs to re-enter the cell cycle in vitro. Although N-SCs are well equal to the task of dedifferentiating to a less mature and more proliferative state following the loss of axonal contact, it is important to
point out that the dedifferentiated SC phenotype is not identical to the immature SC stage of development, as dedifferentiated N-SCs are known to carry a ‘memory’ of their previous differentiated state (Le et al., 2005; Höke et al., 2006; Jessen and Mirsky, 2008, 2010). As such, cultured neonatal N-SCs may represent a less mature population of cells, not only in terms of their actual age, but also in terms of their SC developmental stage.

The notion that neonatal N-SCs have advantages over adult N-SCs still seems rather counterintuitive given that N-SCs from both sources have been reported to elicit reactive astrogliosis, show relatively poor migration/integration into astrocyte-rich territories, and promote only limited axonal growth across the distal graft host interface (Lakatos et al., 2000; Plant et al., 2001; Azanchi et al., 2004; Grimpe et al., 2005; Andrews and Stelzner, 2007; Pearse et al., 2007; Santos-Silva et al., 2007; Schaal et al., 2007). However, to my knowledge, neonatal and adult rodent N-SCs have never been directly compared in terms of their astrocytic interactions, migratory properties or capacities to support axonal growth side-by-side in the same study either in vitro or in vivo. In fact, it is even difficult to meaningfully compare neonatal and adult N-SCs in terms of those properties across studies, as the work examining N-SC interactions with astrocytes in vitro tends to exclusively use neonatal rodent N-SCs, whereas the studies examining the properties of N-SCs transplanted into the injured CNS have largely utilized cells from adult rodent tissue. Furthermore, the few studies that have examined intraparenchymal transplantation of neonatal rodent N-SCs into the injured rat spinal cord (e.g., Li & Raisman, 1994; Keirstead et al., 1999; Azanchi et al., 2004) have generally not included quantifications of astrocyte reactivity, measures of the migration/integration of cells into intact host parenchyma, or axonal growth/sparing assessments that are comparable to other studies transplanting adult N-
SCs (or SKP-SCs for that matter). Thus, although our findings here (Chapter 4) suggest that neonatal N-SCs may have advantages over adult N-SCs, the data that is currently available is inadequate to address the potential differences between those two cell types, so further research will be needed to confirm or refute those results.

5.5 Comparing adult SKP-SCs to adult N-SCs

To date, only two studies have examined SKP-SCs generated from adult rodent tissue sources. The work of Dworski (2011) largely confirmed the similarities between SKP-SCs and N-SCs demonstrated in previous work using neonatal SKP-SCs (Chapter 2, 3, 4; Walsh et al., 2009; 2010; McKenzie et al., 2006), as adult SCs from both sources associated with axons \textit{in vitro} to generate myelinating phenotypes, myelinated the regenerating sciatic nerve \textit{in vivo}, and showed highly similar gene expression profiles. The latter data were perhaps the most striking result from that work, as adult rat SKP-SCs and N-SCs were only found to differ significantly in terms of their expression of 225 genes, or 1.1% of the 20,412 rodent gene probesets. In sharp contrast, 31% of those genes differed significantly when the SKP-SCs were compared to naïve SKPs generated from the same source tissue (adult rat back skin); which indicates that SKP-SCs are far more similar to N-SCs than they are to their own precursor cells when all three are generated from adult rat tissue.

Despite the high degree of similarity in gene expression between the SCs from those two sources, they did appear to form two distinct groups according to hierarchical clustering models. However, with respect to how the two cell types differed, there appeared to be little rhyme or reason, as the vast majority of genes related to SC development did not differ significantly
between the SKP-SCs and N-SCs. Indeed, of the many genes that are known to be relevant to SC development, only 5 had >50% probability of being differentially expressed by adult SKP-SCs and N-SCs, with SKP-SCs expressing higher levels of Pax3, Cdc2, Krox-20 and Id2, and N-SCs expressing higher levels of GFAP. The only recognizable pattern in terms of differential gene expression related to proliferation, as 11 out of 12 proliferation-related genes (e.g., Cyclin D2, Ki67, Pax3, Cdc2 and Id2) analyzed were significantly upregulated by the SKP-SCs; which suggests that those cells were in a more proliferative state than the age-matched N-SCs grown under the same culture conditions.

The work of Assinck et al. (submitted) examined the efficacy of neonatal rat SKP-SCs transplanted into the chronically contused thoracic spinal cord of the rat, and largely confirmed our previous findings transplanting neonatal mouse SKP-SCs under similar conditions (Chapter 2). In addition, that work included a pilot group of two animals that received adult rat SKP-SC transplants. Although statistical comparisons were precluded by the small sample size of that group, qualitative analyses revealed that neonatal and adult SKP-SCs shared virtually identical phenotypes in the injured rat spinal cord. SKP-SCs from both sources survived to a similar extent, partially filled/bridged the lesion cavity in a predominantly rostro-caudal orientation and generated myelinating (P₀⁺) and non-myelinating (p75⁺) SC phenotypes that associated with spared/regenerating axons within the graft and the surrounding spared tissue, while also promoting a robust endogenous SC response. Compared to the results of similar studies transplanting adult N-SCs (e.g., Barakat et al., 2005), both neonatal and adult SKP-SCs appeared to elicit less reactivity from the astrocytes surrounding the lesion site, exhibited greater integration with astrocyte-rich spared host tissue at the rostral and caudal borders of their grafts,
and supported enhanced growth of axons, including SERT- and TH-positive fibres; a small proportion of which appeared to cross the distal graft-host interface. Thus, the results of the preliminary assessment of adult rat SKP-SCs transplanted into sites of thoracic contusion suggested that those cells possess advantages over adult N-SCs in terms of their ability to integrate with astrocyte-rich host tissue and promote axonal growth at the level of the lesion.

Thus at present we know that SKP-SCs generated from adult rodent back skin share a highly similar phenotype with N-SCs generated from adult sciatic nerve, both in terms of their gene expression and their cellular behaviour both in vitro and in vivo. However, it also appears that adult SKP-SCs differ from adult N-SCs in precisely the same ways that neonatal SKP-SCs do, which suggests that SKP-SCs (regardless of source tissue age) have advantages over adult N-SCs following transplantation into the injured CNS, in terms of their ability to integrate with spared host tissue and promote the growth of supraspinal axons, particularly across the distal graft-host interface. In light of the in vivo similarities between neonatal and adult SKP-SCs and neonatal SKP-SCs and N-SCs, it seems likely that both neonatal and adult SKP-SCs may possess phenotypes that are more akin to neonatal N-SCs than adult N-SCs. However, that hypothesis has yet to be tested experimentally, as no direct comparisons between adult SKP-SCs and neonatal SKP-SCs or N-SCs have been conducted thus far.

5.6 Characterizing the SKP-SC phenotype

5.6.1 Do the SKP-SCs represent a ‘less mature’ SC phenotype?

In our initial work transplanting neonatal SKP-SCs, we speculated that the SKP-SCs may have advantages over adult N-SCs because the SKP-SCs are a newly generated SC population
that may be more similar to, or perhaps even contain, SCPs (Chapter 2), whereas the N-SCs represent a more mature, albeit dedifferentiated, SC phenotype. In support of that notion, subsequent studies found that SCPs share similar advantages (less reactive astrogliosis, greater migration and integration, and improved axonal growth) over neonatal N-SCs following transplantation into the injured or demyelinated CNS (Agudo et al., 2008; Woodhoo et al., 2007). However, more recent studies have largely failed to find any evidence that SKP-SCs have a SCP-like phenotype, as neither neonatal or adult SKP-SCs show elevated expression of the SCP-specific marker Cad-19 in comparison to age-matched N-SCs (Chapter 4 and Dworski 2011), and unlike SCPs, here we found that neonatal SKP-SCs do not differ from neonatal N-SCs in terms of the reactivity they elicit from astrocytes, their migration/integration into astrocyte-rich host tissue, or their ability to promote axonal growth at the level of the lesion following transplantation into the injured spinal cord (Chapter 3 and 4). Thus, it would appear that cultured SKP-SCs do not possess an SCP-like phenotype, and although it remains possible that those cells transition through an SCP-like state during their differentiation in vitro, it seems that they do not retain that state, at least when purified and expanded over multiple passages in culture in the presence of NRG1, which is known to drive the differentiation of SCs from SCPs both in vitro (Dong et al., 1995).

If SKP-SCs do not have a SCP-like phenotype, then perhaps their phenotype is closer to that of an immature SC? If that is the case, then those cells would be extremely difficult to distinguish from the N-SCs we harvest from peripheral nerve, as N-SCs take on a dedifferentiated phenotype in the absence of axon contact, and that phenotype is characterized by the downregulation of many genes associated with the mature differentiated state and the
upregulation of a wide array of genes that are expressed by immature SCs during development (Mirsky et al., 2008). Add to that the fact that SKP-SCs generated in culture in the absence of axons are unlikely to precisely match the developmental phenotype of immature SCs, and that both SKP-SCs and N-SCs are generally expanded in culture in the presence of mitogens that are known to alter the expression of genes related to SC development, and it quickly becomes apparent that the differences between SKP-SCs and dedifferentiated N-SCs are likely to be subtle even if SKP-SCs do indeed possess an immature SC phenotype.

In light of these issues, the finding that adult SKP-SCs display enhanced expression of proliferation-related genes, and particularly Pax3 and Id2 (which are both specifically associated with less mature SC developmental states), may be taken as evidence that those cells do in fact represent a less mature SC phenotype than their nerve-derived counterparts. However, the best approach for definitively determining whether SKP-SCs truly represent less mature SCs than N-SCs would be to directly compare these cell types to SCs harvested from less mature peripheral nerve (e.g., SCPs or immature SCs harvested from embryonic nerve). In the absence of such a comparison, or any studies examining the behaviour/effects of immature SCs (harvested from embryonic nerve) transplanted into the injured/intact CNS to compare with the results of similar work transplanting SKP-SCs or N-SCs, it appears that the currently available data are inadequate to address the question of whether SKP-SCs are less mature than their nerve-derived counterparts. Future research should focus on comparisons between neonatal/adult SKP-SCs and N-SCs generated from embryonic tissue if that question is to be properly addressed.

Although we cannot properly address the question of whether SKP-SCs possess an immature SC phenotype given the available data, it is interesting to note that current evidence is
in line with the notion that SKP-SCs may represent a less mature SC phenotype than adult N-SCs, not in the sense that the SKP-SCs belong to a particular stage of early SC development (e.g., SCP or immature SC), but rather in the sense that they are more akin to N-SCs harvested from less developmentally mature peripheral nerve. Here we found that neonatal SKP-SCs share largely similar phenotypes with N-SCs harvested from neonatal nerve prior to the completion of development (Chapter 3 and 4), whereas both neonatal and adult SKP-SCs appear to differ more substantially from N-SCs isolated from adult nerve (Chapter 2; Assinck et al., submitted). Although the neonatal N-SCs we assessed likely include some proportion of immature SCs, that population of cells probably also includes a substantial proportion of dedifferentiated SCs, and therefore does not actually represent a pure population of immature SCs. Thus, a proper comparison of SKP-SCs to immature SCs would still require the isolation of cells from embryonic peripheral nerve.

Other evidence in line with the notion that SKP-SCs are more similar to neonatal N-SCs than they are to N-SCs isolated from more mature peripheral nerve comes from the protein/gene expression studies conducted to date. Although cultured adult SKP-SCs and N-SCs shared highly similar gene expression profiles, Dworksi (2011) did find that those cell types differed in terms of their expression of certain genes related to SC development, including Pax3, Id2, Cdc2, GFAP and Krox-20 (Dworski, 2011). In contrast, cultured neonatal SKP-SCs and N-SCs were not found to differ in terms of their expression of any of those factors in the work presented here (Chapter 4), and given that neonatal and adult SKP-SCs appear to share virtually indistinguishable phenotypes following transplantation into the injured spinal cord (Assinck et al., submitted), it seems likely that adult SKP-SCs will also prove to be more similar to neonatal,
rather than adult, N-SCs. Further research is clearly needed to confirm that theory, as adult SKP-SCs have yet to be directly compared to neonatal SKP-SCs or N-SCs in terms of their protein/gene expression profiles, and more work is needed to confirm the preliminary in vivo findings of Assinck et al. (submitted) and expand that work to include direct comparisons between adult SKP-SCs and neonatal/adult N-SCs.

5.6.2 Do the SKP-SCs possess a phenotype that is more comparable to sensory or motor SCs?

In addition to the phenotypic differences between SCs at different stages of development and those that exist between mature myelinating and non-myelinating SCs, SCs are also known to display different phenotypes based on whether they associate with sensory or motor axons during development in vivo (Höke et al., 2006) and those phenotypic differences are known to persist long-term in culture (Jesuraj et al., 2012). However, to date, none of the comparisons between SKP-SCs and N-SCs have taken the sensory/motor phenotype of the N-SCs into account, as the rodent N-SCs used in the vast majority of studies examining N-SC properties either in vivo or in vitro have been generated from the sciatic nerve, which contains both motor and sensory fibres. Thus, it may be the case that phenotype of SKP-SCs is closer to either a sensory or motor phenotype and we may be none the wiser because we are comparing those cells to a population of N-SCs that contains both of those phenotypes.

With respect to that notion it is interesting to note that compared to motor SCs, sensory SCs are known to display elevated expression of NT3 under baseline conditions and upregulate NGF and BDNF (along with other growth factors) following denervation (Höke et al., 2006). Although it has yet to be shown that N-SCs generated from nerve collected around P1-3 possess
sensory/motor phenotypes, the fact that neonatal SKP-SCs express higher levels of all three of these neurotrophins than neonatal N-SCs from mixed sciatic nerve (Walsh et al., 2009), suggests that neonatal SKP-SCs may possess a phenotype that is more akin to that of a sensory SC. Similarly, although adult SKP-SCs do not appear to differ from N-SCs generated from adult sciatic nerve in terms of their neurotrophin expression, they do express higher levels of numerous genes related to proliferation than adult N-SCs (Dworski 2011), which again suggests a sensory SC phenotype as N-SCs derived from sensory nerve are known to be more proliferative than their counterparts isolated from motor nerve when cultured under similar conditions (He et al., 2012). Thus, there is some evidence to suggest that both neonatal and adult SKP-SCs may possess a phenotype that is closer to that of SC harvested from sensory nerve, but that notion remains to be conclusively tested by comparing neonatal/adult SKP-SCs to age-matched N-SCs generated from purely sensory (e.g., sural nerve) or purely motor (e.g., ventral root) nerves.

This question should be addressed in future work as it may have important implications for the clinical application of these cells. Contrary to the rodent N-SCs used in the vast majority of preclinical work, adult human N-SCs are most often harvested from the purely sensory sural nerve in order to minimize the functional deficits that result from the generation of autologous N-SCs for clinical applications. Thus, if SKP-SCs have a sensory-SC-like phenotype, then one would expect human SKP-SCs to be even more similar to the human N-SCs typically used in the clinic than rodent SKP-SCs are to the rodent N-SCs typically used in preclinical work. In addition, there is evidence that sensory SCs preferentially support the growth of sensory axons and motor SCs preferentially support the growth of motor axons in the injured PNS (Höke et al.,
2006), so the sensory/motor phenotype of human N-SCs and the similarity of SKP-SCs to either of those phenotypes may speak to the capacity of those cells to promote axonal growth in the injured CNS.

5.7 Do SKP-SCs have advantages over N-SCs in terms of their suitability for CNS repair?

To date, all of the studies that have examined the outcome of transplanting neonatal or adult SKP-SCs have found evidence to suggest that those cells display advantages over N-SCs in terms of their cellular behaviour and/or reparative effects in the injured CNS. For example, following transplantation into the injured spinal cord, both neonatal and adult rodent SKP-SCs appear to have advantages over adult rodent N-SCs with respect to the degree of reactive astrogliosis they elicit in the injured CNS, their migration/integration into astrocyte-rich spared host tissue, and their capacity to support the growth of descending serotonergic and noradrenergic brainstem-spinal axon populations, particularly across the distal graft-host interface (Chapter 2; Assinck et al., submitted). In contrast, neonatal rodent SKP-SCs have only demonstrated potential advantages over neonatal rodent N-SCs in terms of the degree of reactive astrogliosis they induce rostral to the site of injury/transplantation and their capacity to promote the growth/sparing of RST axon branches in the gray matter in that region (Chapter 3).

Although those advantages are generally thought to be clinically relevant, as they pertain to the ability of transplanted cells to integrate into spared host tissue and promote axonal growth, it remains unclear whether the differences between these cell types translate into a meaningful advantage for the SKP-SCs over N-SCs in terms of functional recovery. The results of our work
directly comparing the transplanted neonatal SKP-SCs and N-SCs in the injured CNS suggested that the SKP-SCs support greater electrophysiological recovery because the SKP-SC-treated group demonstrated distal forelimb EMG thresholds and latencies that were closer to those of uninjured controls. However, we did not see statistically significant differences between neonatal SKP-SCs and N-SCs on any electrophysiological or behavioural measures following transplantation into the partially injured cervical spinal cord (Chapter 3).

One may expect more robust functional differences between neonatal/adult SKP-SCs and adult N-SCs, given that the SKP-SCs generally have more (and more robust) neuroreparative advantages over N-SCs generated from adult tissue, but the studies transplanting neonatal/adult SKP-SCs after thoracic contusion have generally indicated that those cells promote a degree of functional recovery that is comparable to that typically associated with adult N-SCs transplanted under similar conditions, as SCs from either source generally elicit modest, yet significant improvements in locomotor function relative to media/cellular control treatments (Chapter 2; Tetzlaff et al., 2011; Assinck et al., submitted). However, given the high degree of similarity between those cell types and the lack of robust functional differences in studies directly comparing neonatal SKP-SCs and N-SCs, one might expect the functional differences between SKP-SCs and adult N-SCs to be rather subtle, and therefore difficult to identify by comparing highly variable behavioural data across studies and laboratories. As such I would argue that the true test to determine whether SKP-SCs have an advantage over adult N-SCs has yet to be conducted, because those cell types have never been directly compared in terms of their behavioural or electrophysiological efficacy following transplantation into the injured spinal cord in the same study. Conducting such a study should be a major priority in future research, as it
would not only serve to definitively confirm/reject the purported phenotypic advantages of SKP-SCs over adult N-SCs, but it would shed light on the functional relevance of the neuroreparative differences between those two cell types.

5.8 Are SKP-SCs a suitable alternative to N-SCs for therapeutic application in SCI?

Although I focus a great deal of attention in the present work on examining the differences between SKP-SCs and N-SCs in an effort to determine whether either cell type has any advantages in terms of their suitability as a treatment for SCI, it is important to note that the bulk of the evidence to date indicates that the similarities between those cell types far outweigh the differences.

SKP-SCs and N-SCs generated from rodent tissue respond similarly to known SC mitogens in vitro, proliferating in response to Nrg-1 and upregulating their expression of SC-myelin genes (e.g., MBP, PMP-22, and P₀) in the presence of forskolin (McKenzie et al., 2006). Furthermore, when purified and expanded to confluence in the presence of both NRG1 and forskolin, both neonatal and adult SCs from either of those sources form densely packed swirling parallel arrays of bipolar spindle-shaped cells that label with antibodies raised against characteristic SC markers including S100β, p75, GFAP, O4, or P₀ (Chapter 4 and Dworski 2011). More comprehensive assessments of the protein/gene expression of those cultured cells has revealed that they are exceedingly similar, as neonatal rat SKP-SCs and N-SCs display similar levels of expression of a variety of proteins/genes related to SC development (see Chapter 4) and have thus far only been shown to differ in terms of their production of some neurotrophins (Walsh et al., 2009), whereas adult rat SKP-SCs and N-SCs were only found to
differ in terms of their expression of 1.1% of the over 20,000 genes recently assessed by microarray (Dworski 2011).

Those rodent SKP-SCs and N-SCs have been shown to respond very similarly to axon-derived cues (presumably NRG1) \textit{in vitro}, as SCs from either source will associate with denuded PNS axons, proliferate and differentiate into a myelinating SC phenotype (indicated by the expression of SC-myelin proteins) in co-culture with DRG explants (McKenzie et al., 2006; Dworski et al. 2011). Rodent SKP-SCs and N-SCs also respond similarly to denuded axons \textit{in vivo}, generating myelinating and non-myelinating SC phenotypes (as denoted by morphology and the expression of appropriate SC markers) when transplanted into the injured/dysmyelinated peripheral nerve, the dysmyelinated CNS, or the injured spinal cord (Chapter 2; Chapter 3; McKenzie et al., 2006; Walsh et al., 2009; 2010; Dworski, 2011; Assinck et al., submitted). Importantly, the myelin produced by SKP-SCs and N-SCs appears to be identical, as both cell types ensheath axons 1:1, generate the same complement of SC-myelin proteins (e.g., MBP, P0, PMP-22), and form compact myelin with major dense lines that is indistinguishable from that produced by endogenous SCs (McKenzie et al., 2006).

Rodent SKP-SCs and N-SCs are also highly similar in terms of the reparative benefits they convey in the injured PNS or CNS. Following transplantation into injured peripheral nerve, rodent SKP-SCs and N-SCs are both known to survive, migrate to regions of injury, promote the regeneration of injured axons, and myelinate those regenerating fibres (Dworski 2011; McKenzie et al., 2006; Walsh et al., 2009; 2010). Similarly, following transplantation into the injured spinal cord, both SKP-SCs and N-SCs are known to survive, partially fill/bridge lesion cavities, enhance the sparing of tissue adjacent to the lesion site, increase the presence of endogenous
SCs, myelinate axons, support axonal growth/sprouting into the lesion/graft, and promote functional recovery (Chapter 2; Chapter 3; Assinck et al., submitted).

The high degree of similarity between rodent SKP-SCs and N-SCs, particularly with respect to the benefits they convey following transplantation in preclinical animal models of SCI, supports the notion that SKP-SCs are a suitable alternative to N-SCs for therapeutic application to treat the injured spinal cord. The fact that several SCI transplantation studies have now found evidence to suggest that neonatal/adult rodent SKP-SCs may have advantages over their nerve-derived counterparts also provides support for that notion, because where differences between the two cell types have been found, they invariably favour the SKP-SCs as the preferable cell for CNS repair. However, regardless of whether those differences exist or actually provide any meaningful therapeutic advantage for the SKP-SCs, the preclinical evidence from every injury/transplantation study conducted to date indicates that the SKP-SCs are at least as beneficial a therapy for SCI as N-SCs, and so that data suggests that the SKP-SCs are indeed a suitable replacement for N-SCs in therapeutic clinical applications for SCI. Combined with the fact that the SKP-SCs come from a more accessible source, the harvest of which carries no risk of permanent functional deficits or persistent neuropathic pain and it seems that those cells may not only be suitable, but also preferable, as an alternative to N-SCs in the clinic.

5.9 Towards the clinical translation of SKP-SCs as a treatment for SCI

The overarching goal of our research with SKP-SCs is to determine whether or not those cells represent a suitable replacement for N-SCs as a source of autologous SCs for therapeutic application to treat SCI. That goal can be broken down into two smaller aims: 1) to determine the
efficacy of SKP-SCs as a treatment for SCI; 2) to determine whether those cells are a suitable replacement for adult N-SCs. Towards the first of those aims, we have thus far demonstrated the efficacy of neonatal rodent SKP-SCs as a treatment for moderate thoracic contusion injury in both the sub-acute and chronic settings (Chapter 2; Assinck et al., submitted) and as an acute treatment for discrete unilateral cervical crush injury (Chapter 3). Towards the second aim, we have demonstrated a high degree of phenotypic similarity between SKP-SCs and N-SCs generated from both neonatal and adult rodent tissue sources both in vitro (Chapter 4; Dworski 2011) and in the injured spinal cord; where we have found evidence to indicate that the neonatal SKP-SCs are at least as beneficial as neonatal/adult N-SCs (if not more so) as a treatment for SCI in preclinical animal models (Chapter 2; Chapter 3; Assinck et al., submitted). Thus, the preclinical studies of SKP-SCs conducted to date all support the notion that SKP-SCs are an efficacious treatment for SCI and a suitable replacement for adult N-SCs for those purposes.

However, the true test of the suitability of SKP-SCs as a replacement for N-SCs in the clinic has yet to be conducted, as SKP-SCs generated from adult human skin have yet to be examined in terms of their therapeutic efficacy as a treatment for SCI, let alone compared to their clinically relevant counterparts: N-SCs generated from adult human sural nerve. Those experiments are of the utmost importance in establishing the suitability of SKP-SCs for clinical translation and should therefore be of the highest priority in future research. Although comparisons to human N-SCs are not required to demonstrate the efficacy of human SKP-SCs, evidence that those cell types share extremely similar phenotypes could form the basis of an argument to expedite the translation of human SKP-SCs for clinical application based on the fact
that N-SCs have already demonstrated safety and potential signs of limited efficacy in clinical trials (Saberi et al., 2008; Saberi et al., 2011).

In light of the fact that every study examining rodent SKP-SCs conducted to date has indicated that those cells have advantages over N-SCS, we anticipate similar differences between SKP-SCs and N-SCs generated from human tissue and thus we expect human SKP-SCs to have therapeutically relevant advantages, particularly over their adult nerve-derived counterparts. To date, all that we really know about human SKPs is that cells similar to rodent SKPs can be isolated from adult human scalp and infant/juvenile human foreskin (Toma et al., 2001; 2005; McKenzie et al., 2006), that naïve SKPs from human foreskin are known to respond to NRG1 and forskolin by preferentially generating human SKP-SCs that express S100β, GFAP, p75, MBP and PMP-22 in vitro (Toma et al., 2005; McKenzie et al., 2006) and that those cells generate bona fide SC-myelin when transplanted into the developing CNS (McKenzie et al., 2006). The lack of data regarding the behaviour of transplanted human SKP-SCs thus far has primarily been due to difficulties purifying and expanding those cells, as the protocols that are effective for generating large numbers of SKP-SCs from rodent tissue have proven much less successful when dealing with human skin. However, our collaborators in the Miller (Toronto) and Biernaskie (Calgary) laboratories have recently developed effective protocols to purify and expand human SKP-SCs from adult tissue samples (J. Biernaskie, personal communication), and there are already plans for a number of studies investigating the efficacy of those cells as a treatment for SCI in the near future. Those studies will play a key role in establishing the suitability of SKP-SCs for clinical translation.
5.10 Additional directions for future research

In addition to all of the work comparing neonatal/adult SKP-SCs and N-SCs suggested earlier in preceding sections, and the need to establish the efficacy of human SKP-SCs, particularly generated from adult tissue sources, there are many important questions regarding the properties and efficacy of SKP-SCs that should be addressed in future research.

Thus far SKP-SCs have only been examined in two SCI models (thoracic contusion and cervical DLF crush), so the efficacy of SKP-SCs as a treatment for SCI should be assessed using additional injury models such as clip compression, transection, distraction and/or dislocation injuries. Complete/partial spinal cord transection injuries repaired with biological / artificial conduits or guidance channels would also provide an ideal setting examining whether or not the SKP-SCs have advantages over N-SCs with respect to their capacity to promote axonal growth in the injured CNS, as those models allow clear differentiation between spared and regenerated fibres. Given that SKP-SCs appear to have advantages over N-SCs with respect to their ability to migrate/integrate with astrocyte-rich spared host tissue, future studies should also examine the SKP-SCs as a potential treatment for demyelinating diseases such as MS, by transplanting those cells in EAE, lysolecithin, or cuprizone animal models of demyelination. In addition, it would be interesting to examine SKP-SCs generated from sources other than back skin (e.g., facial or ventral trunk skin), and comparisons between those cells and back skin SKP-SCs and/or N-SCs would be useful in determining whether or not the source of skin alters the phenotype of the SKP-SCs in any way that is pertinent to their application in CNS repair.

Even if SKP-SCs were definitively determined to represent a source of autologous SCs that were suitable as a replacement for N-SCs in SCI therapeutic applications, there would
remain many questions regarding how those cells are best applied, what kinds of injuries they are suited to treat, what co-treatments are appropriate for combinatorial therapies, and what mechanisms underlie the beneficial effects associated with their transplantation. Although a considerable amount of previous research using N-SCs has been conducted to address such questions, there remain many unanswered questions regarding those cells as well, so there is a general need for further examination of autologous SCs as a treatment for SCI, despite the fact that adult N-SCs are already being applied in clinical trials.

For example, given that SCs are primarily intended as an autologous therapy for SCI, the transplantation of cells post-injury should be delayed to take into account the time it would take to isolate, purify and expand those cells from autologous sources in order to maximize the clinical relevance of findings, and yet only two studies have examined the transplantation of SCs at time points that are sufficiently delayed following injury to allow for the generation of cells from autologous tissues harvested post-injury (Barakat et al., 2005; Assinck et al., submitted). In addition, cultured N-SCs have primarily been tested as a treatment for thoracic injuries (Tetzlaff et al., 2011), so there is also a general need to expand the testing of autologous SCs as a treatment for cervical SCI; particularly given that cervical injuries are more prevalent in the clinic (NSCISC, 2012).

Although SKP-SCs may have advantages over N-SCs, neither of those cell types represents a cure for SCI, as much like every other potential treatment tested to date, on their own SKP-SCs only support limited neuroprotection, axonal growth and functional recovery. As such, future work should examine SKP-SCs in combination with other treatments for SCI. Prime examples of potential co-treatments for SKP-SCs include chondroitinase ABC, the elevation of
intracellular cAMP, or the provision of exogenous growth factors (e.g., GDNF); all of which have been found to enhance axonal growth and/or functional recovery in combination with adult N-SC transplantation into the injured CNS (Pearse et al., 2004b; Fouad et al., 2005; Deng et al., 2011; Flora et al., 2012). A variety of previous studies have also used N-SCs themselves as a vector for co-treatment delivery, by genetically modifying those cells to secrete factors that they do not normally produce (e.g., chondroitinase) or over-express specific growth factors (e.g., BDNF and/or NT-3) or cell adhesion molecules (e.g., PSA-NCAM), and those approaches have generally also proven to enhance the therapeutic benefit associated with those cells (Tuszynski et al., 1996; Golden et al., 2007; Ghosh et al., 2012; Kanno et al., 2012). Thus, future research should endeavour to find effective co-treatments to combine with SKP-SCs transplanted into the injured spinal cord, but should also aim to examine the response of SKP-SCs to co-treatments that are known to be effective for adult N-SCs and study the use of genetically modified SKP-SCs.

### 5.11 Concluding thoughts

Throughout most of human history SCI has been considered an untreatable ailment, but the last few decades have finally seen the development of potential therapies aimed directly at treating the neurological damage that underlies the functional deficits associated with that condition. Although none of those therapies have demonstrated sufficient clinical efficacy to be adopted as standards of care for human SCI thus far, it is now widely acknowledged that combinatorial treatment strategies will likely be required to attain that goal, and those strategies
have yet to even reach the clinic. As such, there remains hope for the development of effective treatment strategies for SCI in the future.

The transplantation of autologous SCs remains one of the more promising cellular therapies that might be included in combinatorial strategies in the future, but the best source of tissue to use to generate those cells is currently under debate. As a source of autologous SCs, SKP-SCs have advantages over N-SCs with respect to the ease and safety of their harvest, but a growing body of evidence suggests that those cells may also have advantages over their counterparts generated from adult peripheral nerve; particularly in terms of their abilities to migrate/integrate into spared astrocyte-rich host parenchyma and support axonal growth across the lesion site. If those advantages hold true for human SKP-SCs, those cells should possess an enhanced capacity for CNS repair compared to their nerve-derived counterparts. However, given that the SKP-SCs come from a more accessible and safer source of autologous SCs, adult human SKP-SCS do not need to demonstrate advantages to be considered a suitable alternative for N-SCs generated from adult human nerve, they merely need to match the therapeutic efficacy of those cells. Thus, assuming that human and rodent SKP-SCs share similar properties, it should only be a matter of time before SKP-SCs have been definitively established as the preferred source of autologous SCs for clinical repair of the injured spinal cord.
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