Promoting the survival, migration and integration of Schwann cells by over-expression of polysialic acid

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Promoting the Survival, Migration and Integration of Schwann cells by Over-expression of Polysialic Acid

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A thesis submitted in accordance with the regulations for the degree of PhD

Oct 2010
Abstract

The poor survival and migration of transplanted Schwann cells (SCs) are major drawbacks for their clinical application in cell therapy for neurotrauma. To overcome such drawbacks we genetically modified SCs to over-express polysialic acid (PSA) by lentiviral vectors-mediated expression of polysialyltransferase ST8SiaIV (PST), to study whether over-expression of PSA could enhance their survival, migration, and integration when transplanted into the spinal cord. It was found that more PSA-expressing SCs (PST/SCs) survived than GFP-expressing SCs (GFP/SCs) after transplantation. In vitro expression of PSA on SCs can partially rescue SCs from cell death induced by serum and growth factor withdrawal. In addition, we found high concentration of ATP (>3 mM) could induce SCs death via P2X7 receptor (P2X7R) activation. Blockade of P2X7Rs with an antagonist completely abolished ATP induced SCs death in vitro and also enhance the survival of grafted SCs in vivo. Interestingly, expression of PSA on SCs was found to partially protect SCs from ATP induced cell death in vitro.

PSA expression on SCs did not enhance the motility of transplanted SCs in intact spinal cord. However, in a spinal cord crush injury model PST/SCs transplanted 2.5 mm caudal to the lesion site showed that more cells migrated toward the injury site compared with that of GFP/SCs. Induced expression of PSA in spinal cord further facilitated the infiltration of PST/SCs into the lesion cavity. PST/SCs were also shown to intermingle with the host spinal cells while GFP/SCs formed boundaries with the host tissue. This was confirmed by an in vitro confrontation assay. Furthermore, PST/SCs induced much less expression of GFAP and CSPGs in the surrounding host tissues than GFP/SCs, indicating that expression of PSA on SCs do not cause significant stress response of astrocytes. These results demonstrate that over-expression of PSA on SCs significantly changes their biological
properties and makes them more feasible for cell therapy after neurotrauma.
Acknowledgements

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I need to thank to my parents and my sister, for their continuous encouragement and endless affection. Special thanks to my husband Tao for all his support, encouragement and affection throughout this crucial time of my life.

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Statement of original contributions

This is to claim that all the work presented in this thesis was performed by the author.
List of abbreviations

ALS  amyotrophic lateral sclerosis
AMCA  amino methyl coumarin acetic acid
AMPA  \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATF  activating transcription factor
ATP  adenosine-5\'(-triphosphate
BBG  Brilliant Blue G
BDNF  brain-derived neurotrophic factor
BMSC  bone marrow stromal cell
BrdU  5-bromo-2-deoxyuridine
BSA  bovine serum albumin
BzATP  \(2',3'O-(4\text{-benzoylbenzoyl})\)-ATP
cAMP  cyclic adenosine monophosphate
ChABC  Chondroitinase ABC
CNTF  ciliary neurotrophic factor
CNS  central nervous system
CREB  cAMP response element binding protein
CSPG  chondroitin sulfate proteoglycan
DABCO  1,2, diazabicyclo octane
DC  dorsal column
DCN  dorsal column nuclei
DMEM  Dulbecco's Modification of Eagle's Medium
DNROCK  dominant negative mutant of ROCK
DREZ  dorsal root entry zone
DRG  dorsal root ganglion
DSPG  dermatan sulphate proteoglycan
DTT  dithiothreitol
ECM  extracellular matrix molecule
EDTA  ethylenediaminetetra acetic acid
E  embryonic
Endo-N  endoneuraminidase-N
EPO  erythropoietin
ENV  envelope vector
ER  endoplasmic reticulum
ERK  extracellular signal regulated kinase
ESC  embryonic stem cell
FasR  Fas receptor
FBS  foetal bovine serum
FGF  fibroblast growth factor
FGFR  fibroblast growth factor receptor
FITC  fluorescein isothiocyanate
GAG  glycosaminoglycan
GAP43  growth associated protein-43
GDNF  glial cell-derived neurotrophic factor
GFAP  glial fibrillary acidic protein
GFP  green fluorescent protein
GFP/SCs  GFP-expressing SCs
Glut  glutamate
HCl  hydrochloric acid
h  hour
HBS  HEPES-buffered saline
HGF  hepatocyte growth factors
HRP  horse radish peroxidase
HSPG  heparan sulfate proteoglycan
KSPG  keratin sulphate protoglycan
IFN-γ  interferon gamma
Ig  immunoglobulin
IGF-I  insulin-like growth factor-I
IL  interleukin
iPSC  induced pluripotent stem cell
ir  immunoreactivity
JAK  Janus kinase
LIF  leukaemia inhibitory factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>LV</td>
<td>lentiviral vector</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin associated glycoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NCAM</td>
<td>neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>NDS</td>
<td>normal donkey serum</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NgR</td>
<td>Nogo-66 receptor</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NHS</td>
<td>normal horse serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NPC</td>
<td>neural progenitor cell</td>
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<td>NRG</td>
<td>neuregulin</td>
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<tr>
<td>NSC</td>
<td>neural stem cell</td>
</tr>
<tr>
<td>NT</td>
<td>neurotrophin</td>
</tr>
<tr>
<td>OB</td>
<td>olfactory bulb</td>
</tr>
<tr>
<td>OEC</td>
<td>olfactory ensheathing cell</td>
</tr>
<tr>
<td>OM</td>
<td>olfactory mucosa</td>
</tr>
<tr>
<td>OMgp</td>
<td>oligodendrocyte myelin glycoprotein</td>
</tr>
<tr>
<td>oxATP</td>
<td>oxidized ATP</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PACK</td>
<td>package vector</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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PI 3-K    phosphatidylinositol 3-kinase
PirB      paired immunoglobulin-like receptor-B
PLL       poly-l-lysine
PNS       peripheral nervous system
PST       polysialyltransferase ST8SiaIV
PTPσ      protein tyrosine phosphatase sigma
PSA       polysialic acid
PST/SCs   PSA-expressing SCs
PVDF      polyvinylidifluoride
P2X7R     P2X7 purine receptors
ROCK      Rho kinase
ROS       reactive oxygen species
RT        room temperature
S         second
SC        Schwann cell
SDS-PAGE  sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEMA3     semaphorin 3
SFM       serum free medium
shRNA     short hairpin RNA
siRNA     small interfering RNA
STAT-3    signal transducers and activators of transcription 3
STX       polysialyltransferase ST8SiaII
SVZ       subventricular zone
TE        Tris-EDTA
TEMED     N'-tetramethylethlenediamine
TBS       Tris buffered saline
TNFα      tumor necrosis factors alpha
TRITC     tetramethyl rhodamine isothyocyanate
TTBS      TBS + 0.1% Tween 20
TTX       tetrodotoxin
TU        transduction unit
<table>
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<tr>
<th><strong>TUNEL</strong></th>
<th>terminal deoxynucleotidyl transferase dUTP nick end labelling</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td>vascular endothelial growth factor</td>
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1. Juan Luo, Xuenong Bo, Dongsheng Wu, John Yeh, Peter M Richardson, and Yi Zhang. Promoting the survival, migration, and integration of transplanted Schwann Cells by over-expressing polysialic acid. GLIA (2010, in press)


3. Juan Luo, Yi Zhang, Dongsheng Wu, John Yeh, and Xuenong Bo. Promoting the survival of transplanted Schwann cells by suppressing ATP P2X7 receptors (To be prepared for publication)
Chapter 1
Introduction

1.1 Pathology of spinal cord injury

The pathophysiology of spinal cord injury is best described as consisting of a primary and secondary phase of injury. Primary injury occurs following blunt impact, compression, or penetrating trauma. The immediate mechanical damage to the spinal cord leads to necrosis at the point of impact. Similar to other central nervous system (CNS) trauma, several mechanisms are involved in secondary injury including microvascular alterations; formation of free radicals and lipid peroxidation; accumulation of excitatory neurotransmitters; loss of intracellular balance of sodium, potassium, calcium and magnesium and subsequent increased intracellular calcium level; increased level of opioids, especially dynorphins, at the site of injury (Abraham et al., 2000); depletion of energy metabolites leading to anaerobic metabolism; provocation of an inflammatory response and recruitment and activation of inflammatory cells associated with secretion of cytokines. The secondary injury can cause further tissue damage and activation of calpains and caspas and apoptosis (Knoblach et al., 2005).

1.1.1 Inflammation

The inflammatory process following spinal cord injury is highly complex and involves numerous cellular populations, including astrocytes, microglia, T cells, neutrophils, and invading monocytes. At acute stage, the injury site is rapidly infiltrated by neutrophils from blood which secrete lytic enzymes and cytokines that may further damage local tissue and recruit other inflammatory cells (Popovich et al., 1997). Blood monocytes/macrophages are recruited, as are locally activated resident microglia, both of which subsequently invade to phagocytose the injured tissue (Dusart and Schwab, 1994). These and other reactive cells produce cytokines, such as tumour necrosis factor–α (TNFα), interleukins and interferons, that mediate the inflammatory response and can contribute to further tissue damage (Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Popovich et al., 1997; Donnelly and Popovich, 2008).

Inflammation following spinal cord injury has been revealed as a process of tremendous complexity, with some aspects of the response contributing deleteriously to
further secondary injury, and others contributing beneficially to the removal of cellular debris and enhancement of the environment for regenerative growth (Fleming et al., 2006). Strategies aimed at blocking neutrophil or macrophage influx and at inhibiting of phagocytic and secretory activity of macrophages in the injured spinal cord have resulted in neuroprotection and improved locomotory function (Giulian and Robertson, 1990). However, macrophages and microglia were also shown to promote regeneration of axons by scavenging myelin and neuronal debris (David et al., 1990; Perry and Brown, 1992; Neumann et al., 2009) and producing the proregenerative cytokine, transforming TNFα (Streit et al., 1998). It has been suggested that the better regenerative response after peripheral nerve injury is partially related to the more pronounced macrophage invasion compared that of CNS after spinal cord injury (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998; Kim et al., 2001). TNFα, for example, clearly has beneficial and deleterious effects. The inhibition of TNFα signalling using neutralizing antibodies has been shown to improve functional neurological recovery following spinal cord injury (Bethea et al., 1999). However, TNFα signalling has also been demonstrated to have a neuroprotective role both in vitro (Cheng et al., 1994) and in vivo in TNFα knockout mice which display higher numbers of apoptotic cells, increased lesion size, and worsened functional recovery when compared with the wild-type mice (Kim et al., 2001). Although many cytokines propagate anti-inflammatory response, other cytokines such as interleukin (IL)-10 are considered to have potent anti-inflammatory properties (Knoblach and Faden, 1998). Similar to TNFα, IL-10 is produced by leukocytes, macrophages, astrocytes and microglia, and its administration has been shown to be neuroprotective after experimental spinal cord injury, possibly by inducing anti-apoptotic genes (Bethea et al., 1999; Brewer et al., 1999).

### 1.1.2 Electrolyte imbalances, excitotoxicity and energy metabolism disturbance

Local ischaemia induced by direct injury or vasculature damage around the lesion site causes insufficient oxygen supply. This induces a shift of metabolism from aerobic respiration towards anaerobic glycolysis and leads to an exhaustion of further ATP production and the failure of maintenance of electrolyte balances. The loss of appropriate ionic homeostasis is a central feature of both necrotic and apoptotic cell death following
injury. Regarded to be the most critical among the cations, Ca\textsuperscript{2+} is the trigger for a series of pathological events leading to the failure in neuronal function and spinal shock. Increases in intracellular Ca\textsuperscript{2+} levels can be driven by different pathways. Firstly, Ca\textsuperscript{2+} enters into the cells unspecifically after the initial mechanical insult. Secondly, Ca\textsuperscript{2+} enters due to opening of voltage-gated Ca\textsuperscript{2+} channels after the destruction of cell membrane potential. Thirdly, Na\textsuperscript{+}–Ca\textsuperscript{2+} exchangers are reported to work in a reverse fashion to maintain the usually low intracellular Na\textsuperscript{+} level. More importantly, glutamate activation of N-methyl-D-aspartate (NMDA) receptors allows extracellular Ca\textsuperscript{2+} (and Na\textsuperscript{+}) to move down a massive concentration gradient into the cells. Na\textsuperscript{+} dysregulation has also been implicated in the pathophysiology of spinal cord injury, in particular with regard to damage of the axonal and glial components of the white matter. An intracellular influx of Na\textsuperscript{+} can result from glutamate activation of NMDA, alpha-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) and kainite receptors, as well as from activation of voltage-gated Na\textsuperscript{+} channels and activity of a Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger that shuttles Ca\textsuperscript{2+} out of the cell in exchange for Na\textsuperscript{+}. Whereas action potential propagation normally involves the transient influx of Na\textsuperscript{+} through the activation of voltage-gated Na\textsuperscript{+} channels, the massive depolarization and loss of ATP-dependent ability to move the Na\textsuperscript{+} back into the extracellular compartment lead to a toxic accumulation of Na\textsuperscript{+} and thus water followed into the axon. Animal studies in which ion channel blockers were applied to the spinal cord shortly after injury have highlighted the relevance of dysregulated ionic homeostasis in the pathophysiology of spinal cord injury. Focal microinjections of tetrodotoxin (TTX), a potent blocker of voltage-gated Na\textsuperscript{+} channels, into the injured spinal cord was shown to protect against axonal loss and improve locomotor function (Rosenberg et al., 1999b). In another experiment, treatment with riluzole which blocks TTX sensitive Na\textsuperscript{+} channels or focal injection of TTX, after contusion spinal cord injury in rats helped to reduce loss of primarily white matter and attenuated neurological deficits (Schwartz and Fehlings, 2001). Similarly, glial cell susceptibility to Na\textsuperscript{+} influx through the glutamate activation of AMPA/kainate receptors was alleviated by focal intraspinal injections of 2,3-dihydro-6-nitro-7-sulfamoyl-benzo (f) quinoxaline (NBQX), an AMPA receptor antagonist (Rosenberg et al., 1999a). The acute, time-dependent nature of these processes is highlighted by the demonstration that TTX injections, although effective when performed
within 5 min of injury, lose their effect when applied 4 h after spinal cord injury, which presents a crucial time window for the treatment (Rosenberg and Wrathall, 2001).

Glutamate, acting on both ionotropic and metabotropic receptors, is the most prevalent excitatory neurotransmitter in the CNS. Ionotropic glutamate receptors include the NMDA and AMPA/kainate receptors. Metabotropic glutamate receptors are coupled to G proteins that act as secondary intracellular messengers to mediate a wide spectrum of cellular functions. Glutamate release and accumulation occur rapidly after spinal cord injury in response to ischaemia and membrane depolarization, reaching toxic levels as early as 15 min after experimental injury (Wrathall et al., 1996). The consequence of the activation of NMDA receptors leads to a sharp increase of cytosolic Ca\(^{2+}\) concentrations which are normally extremely low and tightly controlled. Elevated Ca\(^{2+}\) concentrations within the cytosol and mitochondria can trigger a multitude of Ca\(^{2+}\)-dependent processes that can lethally alter cellular metabolism afterwards (Choi, 1987). These processes which include the activation of lytic enzymes, such as calpains, phospholipase A\(_2\) and lipoxygenase; the generation of free radicals and the dysregulation of mitochondrial oxidative phosphorylation lead to the apoptotic death of the cells. Since increased cytosolic and mitochondrial Ca\(^{2+}\) may represent a common final death pathway for numerous insults, NMDA receptor blockade has been extensively evaluated as a potential treatment of spinal cord and other CNS injuries and neurodegenerative disorders.

Adenosine-5'-triphosphate (ATP) is most commonly considered as an energy reservoir, which releases energy during the hydrolysis of its high energy covalent bonds. The roles of ATP had been first suggested as a chemical energy supply for membrane permeability maintenance (Caldwell and Keynes, 1957) and binding to noradrenaline in sympathetic nerves (Schumann, 1958). ATP can be released with noradrenaline, acetylcholine, and other neurotransmitters and the extracellular ATP was found to play important roles in neurotransmission or neuroregulation as a transmitter or a neuromodulator in both the CNS and PNS (Burnstock, 1972; Unsworth and Johnson, 1990; Salgado et al., 2000; Burnstock, 2006, 2007).

The cytoplasmic ATP concentration is in the range 1-10 mM (Beis and Newsholme, 1975). Abundant evidence suggests that ATP is synthesized and stored in vesicles by neurons in CNS and PNS and released in response to depolarisation. ATP can also be
released by glia cells such as astrocytes by mechanical stimulation, although the precise mechanism for the release is still unknown. Following a traumatic injury to CNS, high concentration of ATP can be released at the injury site, from both broken cells and mechanically stimulated cells. Released ATP binds to P2X7 purine receptors (P2X7Rs), a ligand-gated ion channels expressed on spinal neurons and glia cells, and activates several signal pathways and/or induces formation of non-selective membrane pores which lead to cell death. It has been shown that acute transient antagonistic blockade of the P2X7R in a rat model of spinal cord injury decreased cell death in the peritraumatic area of the lesion and led to a significant functional improvement (Wang et al., 2004).

1.1.3 Free radicals and lipid peroxidation
Loss of the Ca\(^{2+}\) homeostasis after injury causes defects in mitochondrial respiratory electron transport. Free radical intermediate products are continuously produced and released. These reactive oxygen species rapidly react with unsaturated and polyunsaturated fatty acid side chains of membrane lipids or with other components of the cells, thus harming the cells and generating more free radicals. Free radical-mediated injury is an important contributor to secondary damage following spinal cord injury with free radical-mediated lipid peroxidation contributing to axonal disruption and the death of both neurons and glial cells. Lipid peroxidation is a self-perpetuating free radical reaction causing membrane damage, leading to cell lysis, dysfunction of organelles, and contributing to Ca\(^{2+}\) dyshomeostasis through the oxidation of membrane lipids. The detection of reactive oxygen species (ROS) peaks at roughly 12 h following injury and remains elevated for 1 week, returning to basal, pre-injury levels 4–5 weeks after injury (Xiong et al., 2007; Donnelly and Popovich, 2008). The production of ROS is associated with both ischaemia immediately following injury and subsequent reperfusion (Sakamoto et al., 1991). Inhibition of free radical generation, particularly relevant to lipid peroxidation, is thought to be one of the principle mechanisms of action for pharmacological agents that have been evaluated for treatment for spinal cord injury. The modest neuroprotective effects of the standard methylprednisolone treatment following spinal cord injury are also believed to be due, at least in part, to the inhibition of lipid peroxidation (Fehlings, 2001; Kwon et al., 2004a).
1.1.4 Cell death and axonal demyelination

Cell death after spinal cord injury may occur by necrosis and/or apoptosis. Traditionally, the death of neurons and glial cells after spinal cord injury was believed to be a passive process resulting from the initial tissue destruction and subsequent inflammatory reactions. In 1995, programmed cell death or apoptosis was first reported after spinal cord injury in rodents (Katoh et al., 1996; Li et al., 1996). Later on, the occurrence of programmed cell death in monkeys and humans was confirmed after spinal cord injury (Crowe et al., 1997; Emery et al., 1998). Typical necrotic cell death involves the swelling of the cells, disruption of organelles, then membrane lysis and release of the intracellular contents that can incite a local inflammatory reaction. Alternatively, apoptotic cell death, or programmed cell death, involves cellular shrinkage with intact organelles and nucleus fragmentation into apoptotic bodies that are subsequently cleared by phagocytosis without a significant inflammatory response. Both necrosis and apoptosis are initiated by similar insults, such as ischaemia, oxidative stress and excitotoxicity, although the more severe the injury, the more likely the cells will undergo necrosis. A cell subjected to massive injury at the site of impact can rapidly succumb to a necrotic type of death. However, cells around the epicenter of injuries that are spared the severe initial trauma may experience a sufficient secondary biochemical insult to initiate an apoptotic programme of self-destruction effected by the subsequent activation of caspases. It is likely that cell death occurs along a spectrum between the two.

Within hours to days after initial injury some neurons and glial cells at the site of the lesion undergo apoptosis. Oligodendrocytes, like neurons, are highly sensitive to ischaemic and traumatic injury (Crowe et al., 1997). The loss of oligodendrocytes results in axonal demyelination, which peaks within 24 h following injury in the rat (Totoiu and Keirstead, 2005). Numerous neurons die in previously unaffected remote areas and axons undergo degeneration afterwards (Crowe et al., 1997; Emery et al., 1998; Li et al., 1999). This process is at least partly dependent on the activation of oligodendrocyte Fas receptor (FasR) or p75 neurotrophin receptor (p75NTR) which in turn activates caspase cascade leading to oligodendroglial cell death (Casha et al., 2001; Beattie et al., 2002; Casha et al., 2005; Chu et al., 2007).
1.2 Current clinical treatments

The priority after spinal cord injury is to stabilize the spine and release the compression of the spinal cord to avoid long-lasting ischaemia. Treatment begins with the emergency medical personnel who make an initial evaluation and immobilize the patient for transport. Immediate medical care within the first 8 h following injury is critical to the patient's recovery. Currently, no gold standard therapy for spinal cord injury has been established. Due to the delayed nature of these events, the secondary phase of spinal cord injury is the most appropriate target stage for therapeutic intervention. Steroids are the main approved neuroprotective drugs which have demonstrated modest, potentially important therapeutic benefits. Corticosteroids have been used in neurotrauma for decades. Their neuroprotective effects include antioxidant properties which are associated with a reduction in TNFα synthesis and nuclear factor κB (NF-κB) activity, enhancement of spinal cord blood flow, reduced calcium influx, reduced posttraumatic axonal dieback, and attenuated lipid peroxidation (Hall, 1992; Oudega et al., 1999). Following the accumulation of preclinical data that are generally supportive of a neuroprotective role for steroids in animal models of acute spinal cord injury (Hall, 1992), methylprednisolone was studied in several prospective acute spinal cord injury trials in humans, making it the most extensively studied drug in acute spinal cord injury (Hurlbert, 2001).

Long-term therapy after spinal cord injury focuses on rehabilitation such as using lower-limb paraplegic cycling, assisted arm-cranking and active treadmill therapy with robotic assistance, pain relief, spasticity treatment, and the prevention of complications. It is well accepted that no single treatment can achieve ideal functional recovery; a combination of various treatments should be considered for individual patients.

1.3 Strategies to promote neural repair

1.3.1 Protecting injury induced neural cell death

Pharmacological intervention

Apart from the administration of corticosteroids described above, a number of promising pharmacological therapies are currently under investigation for their neuroprotective abilities in animal models of spinal cord injury. (1) The Na⁺ channel blocker riluzole was
shown to reduce glutamatergic neurotoxicity, promote the production and release of neurotrophic factors from astrocytes (Peluffo et al., 1997) and markedly enhance the survival of injured motoneurons even when the treatment was delayed (Fumagalli et al., 2006). (2) Minocycline, a synthetic tetracycline derivative commonly used in the treatment of dermatological conditions such as acne and rosacea, has also been demonstrated to have neuroprotective effects in animal models of stroke, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) (Yong et al., 2004). Several laboratories have reported that minocycline attenuated secondary injury and enhanced functional recovery in various animal models of spinal cord injury (Lee et al., 2003; Wells et al., 2003; Stirling et al., 2004; Teng et al., 2004). Its mechanism of action in spinal cord injury appears to be mediated in part by the inhibition of microglial activation, in addition to anti-apoptotic properties (He et al., 2001; Tikka and Koistinaho, 2001; Festoff et al., 2006; Yune et al., 2007). These promising preclinical results have led to two clinical trials, which are now ongoing. (3) The tissue-protective hormone erythropoietin (EPO). It has been shown that EPO can reduce brain injury following a variety of insults (Brines et al., 2000; Digicaylioglu and Lipton, 2001). Evidence shows widespread neuroprotective efficacy of recombinant human EPO in injury models of spinal cord (Celik et al., 2002; Gorio et al., 2002; Grasso et al., 2006). The mechanisms by which EPO exerts its beneficial effects are only partially understood. Available evidence suggests that EPO acts in a coordinated fashion at multiple levels to limit the production of tissue-injuring molecules such as glutamate (Kawakami et al., 2001), reverse vasospasm (Grasso, 2001), attenuate apoptosis (Digicaylioglu and Lipton, 2001; Celik et al., 2002), modulate inflammation (Brines et al., 2000), and recruit stem cells (Shingo et al., 2001). Furthermore, in recent clinical trials of EPO in stroke, it is both timely and prudent to consider the use of this pharmaceutical avenue in traumatic brain injury in humans (Mammis et al., 2009). (4) Cethrin (BioAxone Therapeutic, Inc.), the Rho pathway antagonist, is now under clinical trials investigating the putative neuroprotective and neuroregenerative properties.

**Molecular therapy targeted apoptosis pathway**

Apoptotic death is an active process, both energy dependent and requiring protein
synthesis. It represents a feasible target for therapeutic intervention by selective inhibition (Park et al., 2004). Following spinal cord injury, Fas ligand is expressed by microglia and invading lymphocytes while the FasR is predominantly found on oligodendrocytes (Nagata and Golstein, 1995; Casha et al., 2001, 2005). The interaction of Fas and FasR initiates apoptosis through the activation of the caspase cascade (Nagata and Golstein, 1995). The potential for blocking Fas-mediated cell death following spinal cord injury using a soluble form of the FasR has been demonstrated in vitro and it was found through the intrathecal administration of this soluble form of the FasR, endogenous Fas signalling following spinal cord injury in rats was competitively inhibited in vivo (Ackery et al., 2006; Austin and Fehlings, 2008).

Caspase inhibitors have been tested in several studies. However, the efficacy of caspase inhibitors in counteracting injury induced damage is highly controversial (Li et al., 2000; Ozawa et al., 2002), since these proteases are not the only targets propagating detrimental apoptotic processes. Moreover, caspases also play vital roles in execution of chemotrophic responses of growth cones (Campbell and Holt, 2003). Thus, blocking caspases may also directly interfere with regeneration. Other similar approach includes using inhibitors of calpains that are Ca\(^{2+}\)-dependent cysteine proteases that play an important role in the degradation of cytoskeletal components of damaged cells (Ray et al., 2003). The problems for clinical application, like caspase inhibitors, include the poor penetration to the blood-brain barrier and low cell permeability as well as lack of specificity because many inhibitors also act on other proteases and the proteasome.

The ratio between Bcl-2 and Bax is regarded as a key factor deciding between life and death (Yuan and Yankner, 2000). Over-expression of the anti-apoptotic Bcl-2 in transgenic mice led to less tissue damage after spinal cord injury but failed to bring about any functional improvement (Saavedra et al., 2000; Seki et al., 2003). Similarly, inhibition of the pro-apoptotic Bax, which resulted in strongly decreased oligodendrocyte death after spinal cord injury. However, it could not rescue oligodendrocyte excitotoxic necrosis induced by kainite (Dong et al., 2003). It indicates that targeting cell apoptosis alone may not be enough to rescue cell death after spinal cord injury.
1.3.2 Enhancing the intrinsic regenerative capacity of injured neurons

Delivering neurotrophic factors

Neurotrophins have long been investigated as therapeutics for neurodegenerative diseases. In addition to their involvement in the cell survival, axonal growth and guidance during development, neurotrophins have been shown to regulate neurogenesis and synaptic plasticity in the adult. Neurotrophic factors are released by neurons and glial cells and many of them are up-regulated after injury such as neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) (Hayashi et al., 2000), ciliary neurotrophic factor (CNTF) (Lee et al., 1998) and glial cell line-derived neurotrophic factor (GDNF) (Satake et al., 2000). It has been shown that the successful axonal regeneration in the PNS is partly dependent on the ability of SCs secreting growth factors, supporting axon elongation after nerve injury. Some of the trophic factors that are capable of promoting axon growth after injury include nerve growth factor (NGF), BDNF, NT-3, NT-4, NT-5 and GDNF, with different factors favouring the outgrowth of different populations of neuronal axons (Schnell et al., 1994; Kobayashi et al., 1997; Ramer et al., 2000; Blesch and Tuszynski, 2003; Blesch et al., 2004). For example, the exogenous delivery of NGF in rats can induce extensive sprouting of nociceptive axons (Tang et al., 2004), whereas NT-3 elicits growth of corticospinal axons (Grill et al., 1997). BDNF induces recovery of forelimb function after cervical lateral hemisection and induces axonal growth of rubrospinal, reticulospinal, vestibulospinal, raphespinal, and local sensory and motor axons (Liu et al., 1999; Jin et al., 2000; Jin et al., 2002), and BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rat at T8 level (Vavrek et al., 2006). GDNF induces growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination (Blesch and Tuszynski, 2003). Extracellular signal regulated kinase (ERK) and Akt are two kinases that are activated in response to various neurotrophic factors. Akt also mediates the formation of collateral branches in response to local exposure to neurotrophins (Gallo and Letourneau, 1998; Markus et al., 2002).

The use of trophic factors is considered to be one of the essential components of any combinatorial therapeutic strategy that is used to promote axon regeneration at the site of injury. Several studies have shown the effectiveness of genetically engineered cells
that secrete growth factors, or biomaterials containing trophic factors, in promoting the regeneration and sprouting of injured axons (Lu and Tuszynski, 2008). In addition, delayed delivery of growth factors like BDNF might be less effective than acute delivery because axons of chronically injured neurons may lack appropriate growth factor receptors, such as TrkB receptor which was detected on the neuronal cell bodies, but not on anterogradely labelled rubrospinal axons at the injury site (Kwon et al., 2004b).

**Targeting regeneration associated pathways**

* cyclic AMP

Raising the cAMP level in neurons has been shown to allow them to overcome the inhibitory effects of myelin and myelin-associated glycoprotein (MAG) and promote axon growth (Cai et al., 1999). It has been reported that a stronger regenerative response can be obtained by elevating levels of cAMP in retinal ganglion cells and in the damaged spinal cord (Qiu et al., 2002b; Qiu et al., 2002a; Li et al., 2003b; Rodger et al., 2005). Furthermore, the growth promoting action of conditioning lesions has been mostly attributed to the changes within the cell body of the dorsal root ganglion (DRG) neurons, involving an increase in cAMP concentration in the perikarya in the first days after injury (Qiu et al., 2002b). Elevated cAMP level enhances the regenerative ability of central axons by increasing growth factor receptor like TrkB translocation from the cytoplasm to the cell membrane (Meyer-Franke et al., 1998). cAMP also induces activation of protein kinase A and the subsequent activation of transcription factor cAMP response element binding protein (CREB). This pathway has been shown to be responsible for mediating the neuritogenic effects of neurotrophic factors (Gao et al., 2003).

**Regeneration associated genes**

In addition to cAMP, conditioning lesions in the peripheral branch of DRG neurons also induce the up-regulation of other regeneration-associated genes, such as growth-associated protein-43 (GAP43) (Schreyer and Skene, 1993) and activating transcription factor-3 (ATF3) (Seijffers et al., 2006). GAP43 protein is expressed mostly during development, and is down-regulated in the adult (Skene, 1989); however, it can be up-regulated following injury, especially in the PNS, leading to a successful regenerative response. In contrast to PNS, CNS axons show a reduced ability to upregulate GAP43 in
response to injury (Skene, 1989; Anderson et al., 1998; Rossi et al., 2001). Exogenous expression of ATF3 in DRG neurons promotes neurite outgrowth on permissive substrates (Seijffers et al., 2006), but it remains unclear whether ATF3 over-expression is sufficient to overcome the inhibitory effects of myelin.

There is also another set of genes known to be upregulated after injury. These include those involved in the Janus kinase (JAK)/signal transducers and activators of transcription 3 (STAT3) pathway, through which the neuritogenic effect of cytokines such as leukaemia inhibitory factor (LIF) and IL-6 is mediated. IL-6 administration has been shown to be sufficient for axons to overcome the inhibitory effects of MAG and myelin in vitro and in vivo, and inhibition of the JAK–STAT3 pathway blocks the IL-6 mediated axon regeneration (Cao et al., 2006). However, as a potentially harmful proinflammatory cytokine, IL-6 is upregulated after CNS injury which contributes to increased cell death (Gadient and Otten, 1997).

1.3.3 Neutralizing or blocking axon growth inhibitors

Blocking the effects of myelin related molecules

It has been shown that CNS axons can regenerate when a permissive environment was provided (David and Aguayo, 1981). Products of myelin breakdown and several myelin-associated inhibitory molecules, Nogo, MAG and oligodendrocyte myelin glycoprotein (OMgp) in the adult CNS were thought to be one of the key factors responsible for the failure of axon regrowth (Berry, 1982; Caroni and Schwab, 1988a; Chen et al., 2000; GrandPre et al., 2000). The myelin debris resulting from the injury may pose an important obstacle to the regenerating axons (Filbin, 2003). Nogo, MAG and OMgp are the three major inhibitors associated with myelin.

To date, multiple distinct and quite different molecules have been identified as receptors for MAG, Nogo-66, OMgp, including Nogo-66 receptor 1 (NgR1) and its functional partners p75NTR or TROY, and Lingo-1 (the leucine-rich repeat and Ig domain containing, Nogo receptor-interacting protein-1) (Mi et al., 2004), ganglioside GT1b (Vyas et al., 2002) and most recently paired immunoglobulin-like receptor-B (PirB) (Atwal et al., 2008). NgR1 was initially identified in a screen for Nogo-66 interacting proteins (Fournier et al., 2001). There are two sequence homologues of NgR1 in the
mammalian genome, NgR2 and NgR3, which are expressed in both embryonic and adult PNS and CNS neurons (Xie and Zheng, 2008). Upon ligand binding, the NgR1, p75NTR and Lingo-1 as a single receptor complex triggers intracellular signalling cascades that alter the balance of Rac1 and RhoA signalling. The activity of RhoA is elevated and that of Rac1 decreased, leading to the growth cone collapse and paralysis of growing axons. The activation of RhoA is regarded as a crucial step for myelin inhibitors to exert their inhibitory effect on axon extension. Upon stimulation, RhoA activates its immediate downstream effector Rho kinase (ROCK) to modify actin cytoskeleton dynamics. This intracellular event is a common pathway shared by multiple molecules, including MAG, Nogo, OMgp, Netrin-1, ephrins and chondroitin sulfate proteoglycan (CSPGs) (Yiu and He, 2006); therefore, it can be viewed as perhaps the key convergence point at which various myelin-associated inhibitors exert similar functional outcomes (Lehmann et al., 1999; Dergham et al., 2002; Schweigreiter et al., 2004).

Many attempts were made to block or neutralize the myelin inhibitors. In this regard, strategies for therapeutic intervention include delivering antibodies and peptides to block the binding of inhibitors to the receptor. Significant progress has been made since the first monoclonal antibody IN-1, which acts against Nogo-A protein has been found (Caroni and Schwab, 1988b). Agents presumably work either by binding to all three myelin inhibitors and competing out their function or disrupting the formation of the NgR1/p75NTR/Lingo-1/GT1b signalling complex. More recently, it was found that the soluble extracellular domains of both PirB and NgR1 appear to be much more efficacious at blocking myelin inhibition than antibodies that bind directly to the receptors (Atwal et al., 2008). Another approach is to interfere with the downstream signalling events of the myelin inhibitors. Binding of myelin inhibitors to NgR1 induces protein kinase C (PKC) activation. Blocking PKC activity using inhibitor Go6976 promoted regeneration of the dorsal column fibers (Sivasankaran et al., 2004). RhoA activation is a convergent point in the inhibitory signalling cascade. As the inhibitory interactions between axons and CSPGs may also be in part mediated by the Rho-ROCK pathway (Monnier et al., 2003), it may create potential that targeting Rho-ROCK and its downstream mediators could also have beneficial effects in helping regenerating axons overcome the glial scar. C3 transferase has been used in several studies to inactivate RhoA to promote axonal
outgrowth (Lehmann et al., 1999; Dergham et al., 2002). ROCK can be inhibited with several agents such as Y27632 and it was shown that application of Y27632 both in vitro and in vivo can promote axonal growth (Dergham et al., 2002; Duffy et al., 2009). Our group has investigated whether lentiviral vector (LV)-mediated neuron-specific expression of a dominant negative mutant of ROCK (DNROCK) could promote axon growth. LV/DNROCK was injected into the adult rat red nucleus followed by unilateral rubrospinal tract transection at the C4 level. Rats in the DNROCK group showed better functional recovery in the affected hindlimbs and forelimbs than those in the GFP group. Examination of the spinal cord sections revealed more rubrospinal axonal profiles growing to the spinal cord caudal to the lesion in the DNROCK group than that in the GFP group. These results indicate that blocking the RhoA-ROCK signal pathway by expressing DNROCK can enhance regenerative axonal sprouting and lead to partial recovery of limb function (Wu et al., 2009).

Nogo knockout mice have been generated by several groups independently using different methods to assess the contribution of Nogo to regeneration failure. No consistent and robust regeneration has been reported in mice lacking Nogo (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003; Kim et al., 2004; Zheng et al., 2005). Enhanced axon growth in an OMgp null mutant has been reported, but it was not clear whether the injury models specifically assessed regeneration or sprouting, and the corticospinal tract did not regenerate in this mutant (Ji et al., 2008). For MAG, genetic studies on MAG-/- mice have not implicated any significant role in injury-induced axonal growth (Bartsch et al., 1995). More recently, Lee et al.,(Lee et al., 2010) generated the Nogo/MAG/OMgp triple null mutants. It was found deleting any one of these three inhibitors in mice enhanced sprouting of corticospinal or raphespinal serotonergic axons, there was neither associated behavioural improvement nor a synergistic effect of deleting all three inhibitors. Furthermore, triple-mutant mice failed to exhibit enhanced regeneration of either axonal tract after spinal cord injury. Thus, they suggested that myelin and myelin related molecules might modulate axon sprouting, but did not play a central role in CNS axon regeneration failure. In addition, two groups independently generated and characterized NgR1 null mice. Both groups failed to observe enhanced axon regeneration in the corticospinal tract of NgR1 mutants (Kim et al., 2004; Zheng et
Interestingly, one group found enhanced regeneration of the raphespinal and rubrospinal tracts and improved motor function after complete transection (Kim et al., 2004). The selective enhancement of regeneration in certain axonal tracts in this NgR1 mutant may reflect differences in the intrinsic regenerative capacity of various axonal populations or a differential response of axonal tracts to NgR1 deletion. Regardless what mutation they achieved, the strain genetic background and the induced compensation for other genes need to be considered.

**Modification of glial scar**

It has been known for more than two decades that CSPGs inhibit axonal growth and regeneration. In the adult nervous system, CSPGs are enriched in perineuronal nets, and their abundance is increased in reactive astrocytes following injury to brain or spinal cord. Following spinal cord injury, astrocytes around lesion site display cytotoxic oedema and undergo necrotic cell death from hours to days following injury. A delayed astrocytic response begins in the subacute phase, in which astrocytes become hypertrophic and proliferative, correlating with a dramatic increase in the expression of the glial fibrillary acidic protein (GFAP) (Silver and Miller, 2004). These reactive astrocytes grow multiple, large cytoplasmic processes that interweave to form the glial scar. This scar represents both a physical and chemical barrier to axonal regeneration.

Proteoglycans are a major inhibitory component of the glial scar (Gallo et al., 1987). Astrocytes, as the main cell type of the glial scar, are capable of producing four classes of proteoglycans, including heparan sulphate proteoglycan (HSPG), dermatan sulphate proteoglycan, keratin sulphate protoglycan and CSPA (Johnson-Green et al., 1991). The CSPGs form a relatively large family, which includes aggrecan, brevican, neurocan, NG2, phosphacan and versican. Structurally CSPGs consist of a core protein to which sulfated glycosaminoglycan (GAG) side chains are covalently linked. GAG side chains are traditionally considered the main source of inhibition by CSPGs. More recently, it has been postulated that the protein core also contributes to the inhibition of axonal regeneration by acting as a physical barrier to axon elongation, due to high affinity interactions with other extracellular matrix components such as laminin, fibronectin, and neural cell adhesion molecules (NCAMs) (Yiu and He, 2006). Accumulating data suggest
that CSPGs mediate their inhibitory effect via disruption of integrin function on axons (McKeon et al., 1995; Zhou et al., 2006), and also be in part mediated by the Rho-ROCK pathway (Monnier et al., 2003).

One widely used strategy to overcome the inhibitory effects of CSPGs is to enzymatically digest them in vivo after injury. Chondroitinase ABC (ChABC) is a bacterial enzyme that degrades much, but not all, of the sugar chain from CSPGs, leaving the protein core and stub carbohydrate behind. ChABC is effective at removing the inhibitory properties of CSPGs. Numerous studies have shown that ChABC treatment increased sprouting and improved behavioural recovery after spinal cord injury (Bradbury et al., 2002; Yick et al., 2003; Caggiano et al., 2005; Barritt et al., 2006; Garcia-Alias et al., 2008). To potentially maximize the capacity for axon regeneration and plasticity, ChABC has also been used in combination with transplantation of foetal tissue, Schwann cells (SCs) and/or olfactory ensheathing cells (OECs) or neural stem/progenitor cells (NSCs/NPCs) (Kwok et al., 2008).

Last year, protein tyrosine phosphatase sigma (PTPσ) was identified as the receptor for CSPGs (Shen et al., 2009). Similarly to ChABC treatment, functional ablation of gene encoding PTPσ promoted neurite outgrowth in the presence of CSPGs in vitro and enhanced axonal growth into CSPG-rich scar tissue following spinal cord injury in vivo (Fry et al., 2010). The discovery of neuronal RPTPσ as a receptor for inhibitory CSPGs not only provides important mechanistic clues about CSPG function, but also identifies a potential new target for enhancing axonal growth and plasticity after nervous system injury.

**Targeting other inhibitory molecules in the glial scar**

In addition to the inhibitory effects of CSPGs, several other molecules are now known to be up-regulated after spinal cord injury and to contribute to the growth repulsive effects of the glial scar, such as semaphorin and ephrins.

Meningeal fibroblasts invading the lesion core express the secreted semaphorins Sema3A, Sema3B, Sema3C, Sema3E and Sema3F (Pasterkamp and Verhaagen, 2006). Sema3A which is a major meningeal fibroblast-derived neurite growth-inhibitory factor acts as a chemorepellent through its high-affinity receptor neuropilin 1. Several studies
have shown that after spinal cord injury, expression of Sema3A is increased in fibroblasts that penetrate the lesion deeply, and expression of neuropilin 1 is increased in neurons that project to the site of injury (Pasterkamp et al., 1999; Pasterkamp et al., 2001; De Winter et al., 2002). Sema3A appears to be presented not in a soluble form but as a substrate-bound molecule associated with the cell membrane or extracellular matrix (Niclou et al., 2003). Regenerating axons were excluded from the areas containing Sema3A, essentially creating an exclusion zone at the heart of the lesion. These correlative results indicate that semaphorins help to prevent the penetration of regenerating axons past the centre of CNS lesions. By using a strong and selective inhibitor SM-216289 to Sema3A, it was found that rats with transected injured spinal cord treated with SM-216289 showed substantially enhanced regeneration and/or preservation of injured axons, robust SC–mediated myelination and axonal regeneration in the lesion site, resulting in considerably better functional recovery (Kaneko et al., 2006). Sema4D, a transmembrane semaphorin also known as CD100, is expressed in a subpopulation of oligodendrocytes and is upregulated transiently after spinal cord injury in oligodendrocytes in white matter areas close to the lesion site (Moreau-Fauvarque et al., 2003). Plexin-B1, a Sema4D receptor, displays very limited expression in the adult brain. However, CD72, a Sema4D receptor originally identified in the immune system, is broadly expressed in the mature CNS and could thus mediate the inhibitory effect of Sema4D on adult neurons (Moreau-Fauvarque et al., 2003).

The Eph receptors tyrosine kinases and their ligands, the ephrins, have been implicated in cell migration, neurite outgrowth and axonal pathfinding, topographic mapping, axon fasciculation and vascular formation in the developing nervous system (Klein, 2004; Goldshmit et al., 2006). Several studies have indicated the importance of Ephs and ephrins in the prevention of axon regeneration after spinal cord injury. After spinal cord injury in adult rats, EphA3, A4, A6 and A8 receptors are upregulated on motor neurons as well as astrocytes and oligodendrocytes at the injury site (Willson et al., 2002). It has been shown that the neurite length of wild-type embryonic cortical neurons was significantly longer when these neurons were plated on EphA4-/- astrocytes compared with those plated on wild-type astrocytes. Furthermore, compared to wilde-type neurons, neurite outgrowth of EphA4-/- neurons on EphA4-/- astrocytes was
further increased, indicating that EphA4 expressed on astrocytes and neurons both inhibited neurite outgrowth in response to ephrins (Goldshmit et al., 2004). Expression of EphB3 has also been found on astrocytes at the injury site in the adult rat spinal cord after a complete transection (Willson et al., 2003) or contusion (Miranda et al., 1999). It was proposed that the expression of EphB3 on astrocytes may inhibit regrowth of axons (Miranda et al., 1999). In addition, the inhibitory role of EphrinB2 and its receptor EphB2 in astrocytes and fibroblasts at the site of injury has also been reported (Bundesen et al., 2003).

Fig. 1-1 Glial inhibitors and their intracellular signalling mechanisms.
The molecular inhibitors of the adult CNS glial environment include myelin-associated inhibitors and CSPGs associated with reactive astrocytes in the glial scar. The neuronal receptors and downstream signalling pathways known to be involved in transducing these inhibitory signals are illustrated. (The image is modified from Yiu and He, 2006).
1.3.4 Biomaterials
Lack of extracellular matrix support at the lesion site is considered as one of the factors that hinder the regenerative process after spinal cord injury. Many studies have been conducted to investigate the potential of biomaterials in promoting axonal regeneration in the injured spinal cord, by providing a bridge through which the regenerating axons can be properly guided from one end of the injury to the other end.

Native materials such as collagen, alginate, fibrin and fibronectin, synthetic non-degradable polymers (silicone), synthetic biodegradable polymers such as poly(D,L-lactic-co-glycolic acid), poly(ε-caprolactone), poly L-lactic acid, polyethylene glycol and conducting polymers (polypyrrole, polyaniline) have been used in various nerve regeneration research. An ideal nerve conduit should be thin, flexible, porous, biocompatible, biodegradable, compliant, neuroinductive, neuroconductive and with appropriate surface and mechanical properties (Verreck et al., 2005). Although these biomaterials promise to fulfill some of the above stated criteria, they have some drawbacks which have to be overcome to meet the specific tissue engineering applications. For example, a scaffold made from non-degradable materials should be avoided to prevent the chronic inflammation and compression of the nerve over time. Therefore, it is preferable to use biodegradable materials. However, the advantage of these non-degradable materials over the biodegradable materials is that they do not expose the tissues to the intermediary breakdown products, which may adversely affect the regeneration process (Bakshi et al., 2004). Also, even for biodegradable materials, a surface eroding polymeric scaffold is expected to provide better contact guidance cues continuously for nerve regeneration. Another important property needed for successful graft uptake by host tissues is mechanical stability and compatibility of the scaffolds. For nerve tissue engineering, the scaffold should be pliable, harmless to the surrounding tissues, resist structural collapse during implantation (Bini et al., 2005) which may lead to necrosis and inflammation. Many approaches have been attempted to improve the properties of common biomaterials to make them suitable for neural tissue engineering (Subramanian et al., 2009). Intense investigation has been carried out to improve the neural scaffold properties by several novel fabricating techniques such as polymer
blending and electrospinning, incorporating nerve growth factors or cell graft in the scaffold, and improving the wettability of the scaffold surface by surface modifications.

In general, biomaterial implanted into injured spinal cord showed weak or no axon regeneration (Joosten et al., 1995; Nomura et al., 2006). Thus, combination therapy of biomaterial with neurotrophic factors and/or cell graft is needed.

1.3.5 Cell therapy

Cell replacement strategies have shown much promise for the treatment of neurodegenerative diseases and CNS injuries. The inherent regenerative plasticity of the injured adult spinal cord makes acute spinal cord injury an attractive target for cell replacement strategies. The main goals for the cell based therapy in spinal cord injury mainly are to replace the dead cells, bridge the gap and provide a favorable environment for axon regeneration. The ultimate goal is to achieve reconstruction of appropriate spinal circuits. An important challenge for cell-based therapies is the determination of the optimal cell type, method of delivery, and timing of cellular intervention. Many types of cells have been investigated for their potential for cell therapy for spinal cord injury, including various sources of stem cells, macrophage, fibroblasts, SCs, and OECs.

1.3.5.1 Stem cells

From foetal tissue grafts and bone marrow stromal cells to neural stem cells and embryonic stem cell derivatives, the benefits of stem cell-based transplantation therapies have been well documented in the preclinical studies. Currently, there are several types of stem cells used as cell therapy in spinal cord injury.

**Embryonic stem cell**

Embryonic stem cells (ESCs) are pluripotent cells that have the capability to differentiate into nearly all cell types, including neuronal and glial fate cells (Erceg et al., 2009). Because of their plasticity and potentially unlimited capacity for self-renewal, ESC therapies have been proposed for regenerative medicine and tissue replacement after injury or disease including spinal cord injury. It has been shown that transplantation of human ESC-derived oligodendrocyte progenitors into T9 spinal cord of
dysmyelinated shiverer mice resulted in integration, differentiation into oligodendrocytes, and compact myelin formation (Nistor et al., 2005). In another experiment, human ESC-derived NPCs transplantation into lateral ventricle of the MS mice model significantly reduced the clinical symptoms. Histological examination showed migration of the transplanted cells to the host white matter. However, the differentiation of the transplanted human ESC-derived NPCs into mature oligodendrocytes was extremely rare while the neuroprotective effect benefited from the transplanted cells was observed. This was demonstrated by the suppression effect on the infiltration and activation of T cells (Aharonowiz et al., 2008). Transplantation of ESC-derived NPCs has also been shown to promote regenerative axon growth and neuronal survival by activating BDNF and IL-6 signaling pathways (Glazova et al., 2009). Furthermore, it has been reported that human ESC-derived motoneurons can survive and integrate into the spinal cord (Lee et al., 2007).

However, there are several concerns regarding the efficacy of transplantation of human ESCs in humans. Apart from the ethical concerns, there are some technical issues associated with how to manipulate these cells to differentiate toward defined neural cell types and maintain the same characteristics of the cells in vivo. Moreover, the possible formation of teratomas following the engraftment of human ESC-derived neural cell should not be ignored (Li et al., 2008). Currently, a FDA approved human ESC Phase 1 clinical trial is in progress (Geron Corporation, USA)

**Neural stem cell (NSC) and neural progenitor cell (NPC)**

NSCs are multipotent cells with the potential to differentiate into neurons, oligodendrocytes, and astrocytes. They can be efficiently propagated in vitro (Hsu et al., 2007) depending on the developmental stage and neurogenic zones where the cells were derived, as well as the pathological niche into which they are transplanted (Rossi and Keirstead, 2009). Reynolds and Weiss were the first to isolate NPCs and NSCs from the subventricular zone (SVZ) of adult mouse brain (Reynolds and Weiss, 1992). Since then, NPCs and NSCs have been isolated from various areas of the adult brain, including the spinal cord, and from various species including human (Taupin and Gage, 2002). In the adult CNS, the tissue adjacent to ventricles and the ependymal cells directly lining the
lateral ventricles are known to be rich sources of multipotent NSCs (Johansson et al., 1999). The central ependymal canal of the spinal cord where produces many new progenitor cells that are capable of differentiating toward cells with neural and neuronal characteristics (Thuret et al., 2006).

When cultured in vitro, the NSCs maintain their capacity for self-renewal after several passages and are capable of secreting neurotrophic factors (Coutts and Keirstead, 2008). Functional recovery following NSCs transplantation into spinal cord injury sites has generally been attributed to the remyelination or the release of growth factors from the transplanted cells. However, the generation of astrocytes from NSCs has been reported to be associated with the increased pain (Hofstetter et al., 2005).

It is speculated that NSCs are more favorable than human ESCs for clinical applications because they are considered safer for cell therapy, since NSCs have less potential to form tumours compared with ESCs (Kim et al., 2007). However, many critical challenges remain, including the need for pure populations of differentiated cells, inefficient tracking systems, and moderate cell survival after transplantation (Zhu et al., 2005).

**Mesenchymal stem cell (MSC) and bone marrow stromal cells (BMSC)**

MSCs are multipotent cells that can be derived from many different organs and tissues. They have been demonstrated to play a role in tissue repair and regeneration in both preclinical and clinical studies. Potential advantages of MSCs include the possibility of these cells to be harvested from autologous donors, and relatively rapid expansion in vitro. Thus, they have been identified as promising candidates in the treatment of spinal cord injury.

BMSCs express several types of neurotrophic factors such as NGF, BDNF, GDNF, NT-3, vascular endothelial growth factor (VEGF), and hepatocyte growth factors (HGF) (Chen et al., 2005; Yaghoobi and Mowla, 2006). Moreover, when exposed to an inflammatory environment in vitro, BMSCs alter their growth factor expression profile, indicating that they are responsive to environmental cues (Zhou et al., 2009a). It has been shown that BMSCs transplantation increase axonal growth and plasticity (Cizkova et al., 2006), preserve host tissue and myelinated axons and attenuate cavity formation (Wu et
The mechanisms by which BMSCs exert their effects on the injured spinal cord are likely as a result of expression of neurotrophins.

Both MSCs and BMSCs have potent immunomodulatory properties. Human MSCs inhibit the activation of co-cultured microglia, decrease their production of TNFα and nitric oxide (Zhou et al., 2009a). In a couple of studies, it was found that injection of MSCs inhibited T-cell proliferation and attenuated the release of interferon gamma (IFN-γ) and TNFα, resulting in decreased demyelination, axonal loss and enhanced functional recovery (Zappia et al., 2005; Matysiak et al., 2008; Rafei et al., 2009). Human MSCs have also been shown to reduce astrogliosis and microglial activation while increasing motor neuron survival and motor performance following transplantation into the superoxide dismutase 1 mutant model of ALS (Vercelli et al., 2008).

However, there are several limitations to the current data set that require further investigation. For example, transplantation of different passages of MSCs may result in different outcomes. Another issue that remains to be addressed is the importance of cell dose and whether there is a safe upper limit, especially for long-term effects, including tumor formation, ectopic activity and unwanted fibrosis.

**Induced pluripotent stem cell (iPSC)**

In 2006, Takahashi and Yamanaka showed that mouse embryonic and adult fibroblasts acquired properties similar to those of ESCs after retrovirally introducing genes encoding four transcription factors, namely Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). They called these cells induced pluripotent stem cells (iPSCs). The first generation iPSCs were similar to ESCs in morphology, proliferation, the expression of some ESC marker genes, and the formation of teratomas. From then on, impressive progress has been made in this field.

These cells have been generated from mouse and human somatic cells by overexpression of several defined factors (Ronaghi et al.; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Ronaghi et al., 2010). More recently, generation of iPSCs from human NSCs with a single transcription factor, OCT4, or using direct delivery of recombinant proteins has been discovered (Kim et al., 2009a; Kim et al., 2009b). iPSCs have identical patterns in gene expression, chromatin
methylation, and embryoid body and viable chimera formation (Amabile and Meissner, 2009) as ESCs, thus the potential of iPSCs is considered to be at least equivalent to that of human ESCs. They are capable of differentiation toward all cell types, including glia, NPCs, and motoneurons (Dimos et al., 2008). Furthermore, the derivation of iPSCs using nonviral methods or by chemicals and small molecules makes it very attractive (Huangfu et al., 2008; Kaji et al., 2009; Zhou et al., 2009b). Potential benefits to use iPSC include avoiding immunological rejection after transplantation since these reprogrammed adult cells will be derived from the patients themselves. Also there is no ethical concern for using iPSC which is unlike human embryos.

However, this type of cells shares a similar disadvantage as other stem cell sources, i.e., teratoma formation. There is also unique potential problem to overcome before iPSCs can be used in the clinic, primarily related to the forced reprogramming of somatic cells. It still remains unclear whether nuclear reprogramming is complete for each iPSC clone. Incomplete or aberrant reprogramming of somatic cells to iPSCs could result in impaired differentiation of iPSCs into the required cell type and may increase the risk of immature teratoma formation after directed differentiation (Yamanaka et al., 2000). Another key issue is the presence of transgenes in iPSCs. Transgenes are largely silenced in iPSCs, but the reactivation of such transgenes such as c-Myc could lead to tumorigenesis (Okita et al., 2007). Furthermore, leaky expression of these transgenes may also inhibit complete iPSC differentiation and maturation, leading to a greater risk of immature teratoma formation.

1.3.5.2 Macrophage

Macrophages, which have both beneficial and harmful components, play a critical role in the secondary inflammatory reaction after CNS lesion. Based on transcriptional profiling, proteomics and functional assays, macrophages are typically classified as classically-activated form (M1) or alteratively-activated forms (M2) (Mantovani et al., 2002). M1 macrophages, activated via toll-like receptors and IFN-γ, produce pro-inflammatory cytokines and oxidative metabolites that facilitate their role as indiscriminate killers of microbes and tumor cells. Conversely, M2 macrophages form in the presence of IL-4 and can regulate the inflammatory reaction, clear away dead cells,
promote blood vessels regeneration and tissue re-establishment, and thus provide an environment conducive to regeneration (Gordon, 2003; Mantovani et al., 2004).

Several studies have shown that acute depletion or functional inhibition of macrophages is neuroprotective and promotes recovery of function after spinal cord injury (Popovich et al., 1999; Gris et al., 2004). This is likely due to that microglia and newly recruited monocytes differentiate into proinflammatory M1 macrophages at injured sites (Kigerl et al., 2009). However, early recruitment of blood-borne monocytes at the margins of the lesion site is needed for CNS repair. The blood-borne monocytes acquire a beneficial phenotype only when they encounter the extracellular matrix proteins that delineate the lesion site (Rossignol et al., 2007). It has been demonstrated that timely injection to the margins of the lesion site of specifically activated autologous macrophages at the subacute phase can promote recovery (Schwartz and Yoles, 2006).

The use of autologous macrophages was the first approach developed for clinical trial. Since the preparation of these autologous cells requires only minimal modification, this approach has the advantage of a relatively short development time. In addition, it allows the experimenter to control the number, activity, and site of administration of the injected cells. In a Phase I study, it is shown that autologous macrophage cell therapy is safe and well tolerated in patients with acute spinal cord injury (Knoller et al., 2005). The promising results encouraged the investigators to carry on to Phase II. However, while it was in progress, it was suspended due to a couple of drawbacks in the design of the clinical trial such as the intervention time on patients; the way of cell administration and the high expense involved in the recruitment of each patient.

1.3.5.3 Olfactory ensheathing cell (OEC)

A unique type of cells within the olfactory system is a specialized glial cell called the OEC. OECs have recently attracted much attention and been tested in a few clinical trials in patients with spinal cord injury.

OECs develop from the olfactory placode and subsequently migrate into the olfactory nerve and olfactory bulb. This developmental origin contrasts to that of SCs, which arise from the neural crest. From their origin in the olfactory mucosa to their termination in the glomeruli of the olfactory bulb, the nonmyelinated olfactory nerves are
associated with OECs, which surround large numbers of contiguous axons (Raisman, 1985; Schwob et al., 1992). The olfactory nerve and the outer nerve layer of the olfactory bulb are enriched in OECs, which express p75NTR. The majority of OECs used in axonal regeneration studies on spinal cord injury were from primary cultures obtained from the rat olfactory bulb or olfactory mucosa (Ramon-Cueto et al., 2000; Lu et al., 2001).

Functional recovery and/or CNS axon regeneration has been reported when OECs were transplanted immediately or up to 2 months after spinal cord injury in adult rats (Li et al., 1997, 1998; Lu et al., 2001; Li et al., 2003a). The mechanisms by which OECs promote recovery after spinal cord injury are not clearly known. The growth factors secreted by OECs are also being investigated. In vitro OECs provide both soluble and contact signals to promote sprouting of neurites (Wang et al., 2003; Chung et al., 2004). In vivo, these transplants might also prevent loss of neural tissue (Sasaki et al., 2006) and may enhance myelination after spinal cord injury (Sasaki et al., 2004).

Whether OECs directly myelinate axons after spinal cord injury remains controversial (Boyd et al., 2005). Remyelination by OEC transplants from a variety of species including humans has been reported (Imaizumi et al., 1998; Barnett et al., 2000; Kato et al., 2000). However, some investigators have argued that OECs are not the myelinating cells in those studies but that contaminating SCs are responsible for the remyelination (Rizek and Kawaja, 2006). It has been suggested calponin may be a potential marker expressed by foetal OECs but not adult SCs. They found that OECs, but not SCs, express calponin, a muscle fibre actin-binding protein (Boyd et al., 2006), and that many cells in OEC culture preparations are p75NTR positive and calponin-negative, thus suggesting that OEC cultures were contaminated by SCs. However, in a recent study, it was found that, calponin was strongly expressed by fibronectin-expressing fibroblasts from adult olfactory mucosa, sciatic nerve and skin as well as by meningeal cells from the olfactory bulb, but not by p75NTR and S100 expressing OECs in tissue culture (Ibanez et al., 2007). This data indicates that calponin cannot be used to distinguish adult OECs and SCs. Regardless of the source of transplanted OEC, or whether the myelination observed is primary, secondary or a combination of both, considerable evidence now suggests that CNS remyelination can occur as a consequence of OEC transplantation. OECs are also a promising candidate for combinatorial cell-based strategies (OECs in
1.3.5.4 Schwann cell
SC is one of the most widely studied cell types for repair of the injured spinal cord. These cells play a crucial role in endogenous repair of peripheral nerves due to their ability to dedifferentiate, migrate, proliferate, express growth promoting factors, and myelinate regenerating axons. Following trauma to the spinal cord, SCs migrate from the periphery into the injury site, where they participate in endogenous repair processes. When SCs are transplanted into the lesioned spinal cord they fill cystic cavities and promote axonal regeneration and myelination. More details will be discussed in Section 1.4

1.3.6 Combination strategy
Spinal cord injury incurs damage to axons and successful repair ultimately requires reconnection of the disconnected axons to their appropriate functional effectors. In view of the repair strategy after spinal cord injury, it seems more promising to achieve functional regeneration by manipulating the balance of positive and negative effects of growth-influential molecules in the lesioned spinal cord.

Several studies have shown that combination of autologous BMSCs grafting into the lesioned spinal cord with injection of LV/NT-3 into the host spinal cord rostral to the lesion to establish a NT-3 gradient promoted significantly more ascending sensory axons extended into the host spinal cord rostral to the lesion site (Taylor et al., 2006; Alto et al., 2009). Delivery of ChABC digests restrictive chondroitin sulfate components of the glial scar, resolves scarring with reduction in secondary tissue loss, and increases regrowth of myelinated axons across the lesion gap. Massey et al. (Massey et al., 2008) found that application of ChABC or LV/NT-3 in the rat dorsal column nuclei (DCN) promoted ingrowth of some axons of transplanted DRG neurons. When the two treatments were combined, entrance by regenerating axons into the DCN was significantly augmented. Administration of cAMP or rolipram, the phosphodiesterase 4 inhibitor that prevents the breakdown of cAMP, induces injured axons/neurons to overcome myelin-associated inhibitory signals (Hannila and Filbin, 2008). In the Miami Project to Cure Paralysis, it
was found the most efficacious strategy for the acute complete transection/SC bridge model, including improvement in locomotion was the combination of SCs, OECs, and chondroitinase administration; the most successful combination strategy for a subacute spinal cord contusion injury to be SCs and elevation of cAMP via subcutaneous infusion of rolipram combined with direct injection of an analogue of cAMP, dibutyryl cAMP above and below the SC implant (Bunge, 2008). These results demonstrate that development of the right combination strategies that have significant synergistic effect may hold the key to achieve successful axon regeneration.

An optimal recovery of specific skills was observed recently when a treatment with ChABC was combined with a task specific training. It seems that promotion of plasticity with chondroitinase opens a time window during which rehabilitation becomes more effective. However, it is noteworthy that only trained skills were improved whereas others could be negatively affected compared to the effects of chondroitinase treatment alone (Garcia-Alias et al., 2009). Combined with Nogo neutralization in partially lesioned rats, intensive training seems to interfere with functional recovery, suggesting competition between the underlying mechanisms when both strategies are applied together (Maier et al., 2009), but training starting one month after OEC transplantation in transected rats improves recovery (Kubasak et al., 2008). This shows that the timing for the application of the different strategies is crucial.
Fig. 1-2 Diagram shows the strategies to promote axonal regeneration. (The image is modified from L. McKerracher and S. David, 2004).

1.4 Schwann cell for neural repair
1.4.1 Characterization of Schwann cells
SC is named in honor of the German physiologist Theodor Schwann (1810-1882) who is now acknowledged as the founder of modern histology. Two intermediate cell types are known to be involved in the generation of SCs from neural crest cells. The first, the SC precursor, is found in rat peripheral nerves at E14 and 15 (mouse E12 and 13). The second, the immature SC, is present from E17 (mouse E15) to around birth. At this time, the immature cells start to differentiate. Myelinating SCs mature first, and the non-myelinating cells appearing later (Mirsky et al., 2002). Even though all SCs are of neural crest lineage, SCs in the mature PNS can be further classified by their morphology, antigenic phenotype, biochemistry and anatomical location. These categories are (1) myelinating SCs, (2) non-myelinating SCs, (3) perisynaptic SCs at the neuromuscular junction where they not only enwrap the axonal length but also extend processes to encompass the synapse (Corfas et al., 2004) and (4) satellite cells that are found in the
DRG and ensheathe the cell bodies of sensory neurons (Hanani, 2005).

In a mixed peripheral nerve unmyelinated fibres vs. myelinated fibres by a ratio of three or four to one (Jacobs and Love, 1985). In rodents, the postnatal formation of myelinating and non-myelinating cells is a slow process, taking several weeks to complete. During the early postnatal period, immature SCs diverge, generating myelinating cells that wrap the large diameter axons and non-myelinating cells that accommodate small diameter axons in shallow troughs along their surface. Although the physiological roles of non-myelinating SCs are poorly defined, it is becoming apparent that they are vital for the function and maintenance of unmyelinated axons and also necessary for pain sensation (Chen et al., 2003).

Following nerve transection, mature SCs promptly undergo radical changes in morphology and gene expression leading to developmental regression of individual SCs and myelin breakdown. In nerves, these processes are accompanied by SC proliferation (Fawcett and Keynes, 1990). The eventual outcome is the generation of a single population of cells that are comparable, although not identical, to immature SCs in neonatal nerves (Jessen and Mirsky, 1992). Such cells provide an environment particularly conducive to axonal re-growth, which is probably related to their relatively high level expression of neurotrophic factors and cell adhesion molecules (Mirsky et al., 2002).

1.4.2 Advantages of using Schwann cell in neural repair
SCs have several unique features making them one of the best cell types for therapy. Firstly, they express a variety of factors that support the growth of central axons. Among them are members of the neurotrophin family including NGF, BDNF, and NT-3, as well as CNTF, GDNF, and fibroblast growth factor (FGF). Secondly, SCs express surface molecules such as L1/Ng-cell adhesion molecules and NCAM (Martini and Schachner, 1988), both of which have been demonstrated to support axon growth through contact guidance (Lemmon et al., 1989; Cremer et al., 1997). Finally, SCs produce axon growth-promoting substrates such as laminin, fibronectin, and collagen (Oudega and Xu, 2006). One principal advantage of SCs over other cell types for transplantation into the injured CNS is that SCs are able to myelinate both regenerating and intact central axons.
(Oudega and Xu, 2006; Lavdas et al., 2008). Many other cell types promote axonal regeneration, but do not form myelin sheaths around the new axon sprouts. SCs do so and thus facilitate signal conduction in the regenerated axons.

To employ SCs for transplantation into the injured CNS, obtaining large numbers is necessary to fill up large and numerous cystic cavities developed following the initial injury. The development of in vitro systems to harvest and expand human SCs presents a unique opportunity for autologous transplantation (Casella et al., 1996; Levi, 1996). Importantly, the cultured SCs were shown to retain their ability to myelinate and promote axonal regeneration. A new approach of isolating skin-derived precursors and differentiating the cells into SC progeny may provide another source of SCs for transplantation (Toma et al., 2005; Biernaskie et al., 2006; McKenzie et al., 2006). Recently human MSC-derived SC-like cells were also transplanted into spinal cord and were shown to promote axonal growth (Park et al., 2010).

The efficacy of SCs in promoting axonal regeneration and myelination in the injured adult mammalian PNS and CNS has been extensively studied in a variety of experimental models (Oudega and Xu, 2006; Fortun et al., 2009). SCs transplantation in injured peripheral nerve, optic nerve and spinal cord promoted axonal regeneration, and ensheathed or myelinated the regenerated axons (Oudega and Xu, 2006; Fortun et al., 2009). In cases where axons in the spinal cord were demyelinated but not severed, remyelination by grafted SCs re-established normal conduction velocity across the lesion and thus achieved functional repair. Moreover, SCs mixed with Matrigel, when seeded into semipermeable polycrylonitrile/polyvinylchloride polymer tubes then grafted between stumps of the completely transected (Xu et al., 1997) or hemisected (Xu et al., 1999) adult rat thoracic spinal cord have been shown to promote propriospinal and supraspinal axonal growth across the gap and myelination. The cord stumps were bridged by a tissue cable that contained SCs and axons were at different stages of myelination or ensheathment by SCs (Xu et al., 1995b; Xu et al., 1995a; Xu et al., 1997). The absence of SCs in control grafts resulted in scarce axonal growth. Moreover, SC can be genetically modified to over-express neurotrophic factors such as BDNF and NT-3. Behavioural assays (Rotarod assay and Grid walk assay) showed that NT-3- and BDNF-transduced SCs promoted faster and stronger functional recovery than GFP-transduced SCs when
they were transplanted into demyelinated spinal cords of adult mice induced by lysophosphatidylcholine injection. Moreover, NT-3-transduced SCs provided neuroprotection and reduced astrogliosis (Girard et al., 2005).

1.4.3 Limitations of using Schwann cell in neural repair

Although SCs transplanted into the CNS can promote axon regeneration, remyelination and improve functional recovery in animal models of spinal cord injury, poor survival of transplanted SCs in the CNS is a common problem and limits their therapeutic potential. Lack of matrix support, serum and trophic factor withdrawal and inflammation have been shown to induce cell death (Li et al., 2003c; Hill et al., 2006). Though it still remains unclear whether this is the key factor that can cause much difference for the final functional recovery, the predominant idea is that enhanced survival of transplanted grafts will benefit the ultimate outcome.

To address their nature as PNS neuroglia which is distinct from CNS neuroglia, it needs to be aware of the limited integration of transplanted SCs. It was reported that SCs, when in contact with astrocytes in vitro, could induce stress response of astrocytes (Lakatos et al., 2000; Fairless et al., 2005), i.e.: boundary formation and astrocyte activation (increases in cytoplasmic area and expression of both GFAP and CSPGs (Wu et al., 1998; Lakatos et al., 2000). This is further confirmed by in vivo SCs transplantation experiment (Lakatos et al., 2003b).

Taken together, the poor survival and lack of integration ability with host tissue are two key issues need to be solved. Overcoming these obvious disadvantages is critical before SCs can be used for efficacious therapeutic intervention. One of the solutions is to genetically modify SCs in vitro by long-term and stable expression of growth-promoting or inhibitor-blocking molecules or agents.

1.5 Potential factors responsible for the death of transplanted Schwann cells

1.5.1 Serum and growth factor withdrawal

SCs underwent apoptosis in vitro upon serum withdrawal for 48 h (Syroid et al., 1996). Evidence suggested that lysophosphatidic acid (1-acyl-2-sn-glycerol-3-phosphate) (LPA)
could be a potential candidate for mediating this survival effect of serum. LPA is present in serum at micromolar concentrations. Furthermore, LPA receptor, LPA1, is highly expressed in SCs throughout sciatic nerve development and in cultured SCs (Eichholtz et al., 1993; Weiner and Chun, 1999; Weiner et al., 2001). It was found 60–75% of SCs underwent apoptosis by 48 h of serum withdrawal, and up to 80–90% by 72 h. Under serum deprivation, LPA at concentrations as low as 10 nM significantly rescued SCs from apoptosis and the effect was concentration-dependent. The reduced cell death following LPA treatment was the result of cell survival rather than cell proliferation, as BrdU incorporation was not increased by LPA treatment (Weiner and Chun, 1999). Furthermore, it was found LPA had comparable efficacy in promoting SC survival as neuregulin (NRG), another potent SC survival factor. Treatment with either molecule was observed to inhibit around 50% of the apoptosis induced by serum deprivation, suggesting that they may activate convergent singalling pathways (Ye et al., 2002).

A number of growth factors influence SCs development and death, including NRGs, transforming growth factor-β, FGF, PDGF, and insulin-like growth factor-1 (IGF-1) (Eccleston et al., 1993; Dong et al., 1995; Gavrilovic et al., 1995; Syroid et al., 1996). SCs express receptors for these trophic factors, including the PDGF and NRG receptors, and the type-1 IGF receptor (IGF-1R) (Eccleston et al., 1993; Leventhal et al., 1995; Grinspan et al., 1996; Carroll et al., 1997).

Basic fibroblast growth factor, also known as bFGF, FGF2 or FGF-β, is a member of the fibroblast growth factor family. bFGF is available during peripheral nervous system development and regeneration as neurons synthesize bFGF (Eckenstein et al., 1994) and increase bFGF expression after axotomy (Ji et al., 1995). SCs express bFGF receptor (Neuberger and De Vries, 1993a, b), a receptor with endogenous tyrosine kinase activity (Ullrich and Schlessinger, 1990) and respond to exogenous bFGF by activation of cellular division (Raff et al., 1978). In addition to its mitogenic effect on SCs (Chen et al., 1991), bFGF prevents cAMP-induced apoptosis in cultured SCs (Shaw et al., 1997).

IGFs, including IGF-1 and IGF-2 are trophic factors whose growth-promoting actions are mediated via IGF-1R and modulated by six IGF binding proteins. IGF-1 and IGF-1R are present in SC and axons in developing nerves, reaching peak levels during periods of myelination (Cheng et al., 1999). It was found when SCs were switched to
serum-free defined media for 12–72 h, they underwent apoptosis. Addition of insulin or IGF-1 prevented apoptosis. The phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 blocked IGF-1–mediated protection and Akt, a downstream kinase of PI-3K, mediate this effect. It was found in dominant negative K179M Akt (K179M) transfected SC, Akt is not activated in response to IGF-1. In addition, IGF-1 is unable to promote SC motility and survival in K179M-SC which suggest a critical role for Akt in IGF-1 mediated motility and survival in SC (Syroid et al., 1999). In animal studies, there is a significant increase in IGF-1 expression in injured peripheral nerves (Glazner and Ishii, 1995). In the CNS, IGF-1 is the only growth factor that promotes oligodendrocytes to proliferate, differentiate, and myelinate (McMorris et al., 1993). In mice that were genetically altered to over express IGF-1, brain mass was increased because of enhanced myelin production by oligodendrocytes (Carson et al., 1993).

β-Neuregulin (βNRG) is a potent SC survival factor that binds to and activates a heterodimeric ErbB2/ErbB3 receptor complex. It was found that NRG receptor singalling rapidly activated PI3K in serum-starved SCs, while PI3K inhibitors markedly exacerbated apoptosis and completely blocked NRG-mediated rescue. NRG also rapidly signaled the phosphorylation of mitogen-activated protein kinase (MAPK) and the serine/threonine kinase Akt. The activation of Akt and MAPK in parallel pathways downstream from PI3K resulted in the phosphorylation of Bad at different serine residues. As mentioned above, several factors that rescue SCs from apoptosis have been identified, including bFGF, IGF, and the βNRG. The survival of early SC precursors was shown to be dependent on FGF-2 and IGF, but NRG becomes a predominant survival factor as the SCs mature (Jessen et al., 1994; Dong et al., 1995; Gavrilovic et al., 1995). An autocrine mechanism emerges during the postnatal period when SCs begin secreting their own survival factors, including NRG and IGF-1 (Cheng et al., 1998; Syroid et al., 1999). In addition to survival, NRGs mediate SC differentiation, proliferation, and migration through binding and activation of a heterodimeric ErbB2/ErbB3 coreceptor (Grinspan et al., 1996; Vartanian et al., 1997).

Forskolin increases intracellular cAMP levels and is implicated in SC proliferation (Jessen and Mirsky, 1992). It was shown forskolin promoted survival after transfer of SCs from serum to serum-free medium (Cheng and Mudge, 1996). However, in another study,
it was found forskolin itself had no effect on survival of SCs in serum-free medium, but it enhanced the effect induced by the other growth factors (Cheng et al., 1998).

1.5.2 Glutamate
Glutamate is the principal excitatory neurotransmitter. The two main types of glutamate receptors are ionotropic and metabotropic. Ionotropic receptors are directly coupled to membrane ion channels. The metabotropic receptors are coupled to intermediary compounds, such as G protein, and modulate intracellular second messengers, such as inositol-1,4,5-trisphosphate, calcium, and cyclic nucleotides. The directly coupled ionotropic receptor can be further subdivided into three subtypes: NMDA, AMPA and kainate. The NMDA receptors activation allows the influx of extracellular calcium (and sodium followed). Excessive accumulation of intracellular calcium is the key observed process leading to neuronal death or injury.

Normal extracellular glutamate concentration is about 0.6 µM (Bouvier et al., 1992). Substantial neuronal excitotoxic injury occurs with glutamate concentrations of 2 to 5 µM (Meldrum and Garthwaite, 1990; Rosenberg et al., 1992). Traumatic injury to neurons can release the intracellular glutamate to the extracellular space, with concentrations reaching to about 10 µM. One recent therapeutic strategy is to immediately treat patients with injuries to the head or spinal column with glutamate receptor blockers to minimize the spread of neuronal death beyond the immediate physically disrupted neurons (Muir, 2006). Pharmacological study has shown NMDA receptors localized on SCs from guinea pig (Fink et al., 1999)

1.5.3 ATP
1.5.3.1. Biological functions of extracellular ATP and purinoceptors
The concentrations of ATP in cells are in the range of 1-10 mM (Beis and Newsholme, 1975). Upon cell breakage after injury, intracellular ATP will be released and the local concentration of ATP could be in mM range. Extracellular ATP can be degraded to ADP by ectoATPase. During cell transplantation procedure, although it is done very carefully to minimize the damage to the host tissue, certain injury is unavoidable due to penetration of injection needle. Also the space occupied by transplanted cells will put pressure on the
surrounding host tissues which may trigger the release of ATP from astrocytes, which has
been shown to release large amount of ATP even by mechanical stimulation (Newman,
2001).

The extracellular effects of purines (and some pyrimidines as well) are mediated by
purinoceptors, which has been classified into P1 and P2 families (Burnstock and
Kennedy, 1985). P1 (also called adenosine receptors) consists of A1, A2a, A2b, and A3
subtypes, is sensitive to adenosine and AMP. P2 purinoceptors are subdivided into P2X
(ligand-gated ion channels) and P2Y (G-protein-coupled metabotropic receptors)
subfamilies, which are activated by ATP, UTP, or ADP. Seven subtypes of P2X and eight
subtypes of P2Y receptors have been cloned (Abbracchio et al., 2009). Each subtype of
purinoceptors showed different affinities to different agonists and antagonists. Many of
the P2X subtypes can form heteromultimers and gain new biophysical properties.

1.5.3.2. Expression and biological functions of P2X7R
Special attention has been paid to P2X7R subtype due to its involvement in inflammation
and immune response. The P2X7R is highly expressed in macrophages, monocytes and
lymphocytes (Surprenant et al., 1996). Within the CNS, functional P2X7R is localized on
microglia and astrocytes (Ferrari et al., 1996; Collo et al., 1997; Sim et al., 2004). In rat
DRG, P2X7R appears to be selectively localized on glial cells, but not neurons (Zhang et
al., 2005). However, the existence of functional P2X7R on peripheral or central neurons
remains controversial due to the poor specificity of both antibodies and ligands targeting
the rat P2X7R (Sim et al., 2004; Anderson and Nedergaard, 2006). Unlike other members
of the P2 receptor superfamily, homomeric P2X7R is activated by high concentrations of
ATP (> 100 µM) and 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP). BzATP is the most
potent agonist for P2X7R, though it is not a selective agonist for this receptor (Jacobson
et al., 2002). Brief activation of P2X7R by ATP results in Na⁺ and Ca²⁺ influx, K⁺ efflux,
membrane depolarization. During continued or prolonged application of agonist, the
P2X7R channels can aggregate to form large pores that allowed large molecules such as
YO-PRO to pass through them (Surprenant et al., 1996). Membrane blebbing, cell
apoptosis, inflammatory cytokine and chemokine expression are also involved in the
P2X7R activation (Donnelly-Roberts and Jarvis, 2007).
It has been reported that the activation of P2X7R was related to the process of cytokines singalling (Solle et al., 2001). The leukocytes from P2X7R knockout mice were found having abolished responses to ATP, such as the expressions of L-selectin and IL-1β. Additionally, the P2X7R knockout mice had markedly less incidence and severity of monoclonal anti-collagen-antibody induced arthritis compared to that of the wild-type mice. These data indicated the P2X7R can be seen as a crucial component of an in vivo inflammatory response (Labasi et al., 2002).

The finding that disruption of P2X7R not only altered inflammatory pain but also reduces pain associated with frank nerve injury (Chessell et al., 2005) provided new insights into the potential physiological role of P2X7R in sensory functions. Previous data have shown that endogenous IL-1 levels were increased in the nervous system in response to trauma associated with mechanical damage, ischaemia, and hyperexcitability (Touzani et al., 2002). Increased IL-1 levels are also associated with enhanced nociceptive singalling in a concentration related fashion (Bianchi et al., 1998). Blockade of IL-1 receptors with the IL-1 receptor antagonist in spinal cord resulted in reduced nociception in animal models of inflammation and nerve injury induced pain (Safieh-Garabedian et al., 1995; Sommer et al., 1999).

It is well established that ATP acting at P2X7R serves as an efficient secondary stimulus for the maturation and release of IL-1β from microglia and macrophages (Perregaux and Gabel, 1994; Ferrari et al., 2006). Thus, P2X7R activation may act as a danger signal in the context of tissue injury (Ferrari et al., 2006). However, due to the previous lack of appropriate pharmacological tools, the progress in disease models was quite slow.

1.5.3.3 P2X7R activation and cell death
Prolonged (>15 min) ligation of the P2X7R is reported to cause cell death and dramatic morphology changes in microglia/thymocytes/macrophages (Hogquist et al., 1991; Perregaux and Gabel, 1994; Falzoni et al., 1995; Ferrari et al., 1999; Tsukimoto et al., 2005). More recently, it was found P2X7R was expressed in hematopoietic stem cells/progenitor cells, and P2X7R activation was involved in the cell apoptosis induced by ATP (Yoon et al., 2007; Delarasse et al., 2009).
Severe retardation of cell proliferation and cell apoptosis were observed following high-dosage ATP stimulation. The activation of intracellular caspases by the P2X7R is required for apoptotic alteration of ATP-induced cell death (Ferrari et al., 1999; Yoon et al., 2007; Delarasse et al., 2009). In another study investigating the detail mechanism of ATP induced cell death, it was found the BzATP-induced cell shrinkage was blocked when media Cl\(^-\) was replaced with gluconate. Removal of extracellular Cl\(^-\) blocked the BzATP-induced lactate dehydrogenase release, but not the pore formation and the membrane depolarization, which indicates that removal of extracellular Cl\(^-\) did not prevent BzATP from binding to P2X7R but affected the process of apoptotic cell death. Taking the involvement of pore formation in cell shrinkage into consideration, they supposed that the involvement of extracellular Cl\(^-\) influx via the pore formed in the P2X7R-mediated apoptotic cell death (Tsukimoto et al., 2005).

P2X7R has been shown to be localized on mouse SCs by electrophysiology and immunocytochemistry (Colomar and Amedee, 2001). However, no direct study shows whether P2X7R activation induce SC death. If P2X7R is involved in the SC death during/after transplantation when high concentration of ATP is released, targeting P2X7R may enhance the survival of transplanted cells.

1.6 Polysialic acid (PSA)

1.6.1 Structure and biophysical properties of polysialic acid

PSA is a linear polymer of N-acetylneuraminic acid in \(\alpha2,8\)-linkage (Finne et al., 1983). It is mainly attached to the two asparagines in the Ig5 domain of the extracellular part of NCAM via a typical N-linked glycan (Hildebrandt et al., 2008). A single PSA chain may consist of more than 50 monomers, but the chain length may vary substantially (from 8 to 100 monomers) (Inoue and Inoue, 2001; Galuska et al., 2006). These variations occur during developmental stage, between different regions of a tissue, and even on an individual neuron. Polysialylation can add 30% of relative molecular mass to NCAM molecules. PSA is mainly detected on NCAM, but is also found on the \(\alpha\) subunit of sodium channels in adult rat brain (Zuber et al., 1992) and on neuropilin-2 of human dendritic cells (Curreli et al., 2007).
Fig. 1-3 Schematic representation of the structures of cell adhesion molecule NCAM. The three main isoforms of NCAM have five Ig-like domains and two fibronectin III repeats. NCAM-120 lacks a cytoplasmic domain and is attached to the membrane via a glycosylphosphatidylinositol link. Two polysialic acid chains can be attached to the fifth Ig domain of the NCAM molecule. (The image is modified from H. Hildebrandt et al., 2008).

PSA is negatively charged and can occupy large space when hydrated (Yang et al., 1992; Yang et al., 1994; Rutishauser, 2008). Electron microscopy images show that PSA expression increases intercellular spacing by 10–15 nm (Rutishauser et al., 1988). By directly measuring the repulsive and adhesive forces between two lipid membranes with membrane-bound NCAM, PSA-NCAM, or cadherin, it has been shown that NCAM polysialylation increases the range and magnitude of inter membrane repulsion (Johnson et al., 2005). The repulsion can attenuate not only homophilic NCAM–NCAM binding but also affects heterophilic binding with other cell surface molecules such as L1, laminin, cadherin, and integrin (Rutishauser et al., 1988; Acheson et al., 1991; Rutishauser and Landmesser, 1996; Johnson et al., 2005). Those interactions do not appear to depend directly on NCAM’s intrinsic binding function, because they are unaffected by NCAM cytoplasmic domain splicing variations and deletion of NCAM Ig 1–4 domains (Fujimoto
et al., 2001). Instead, the interactions are linked to PSA steric repulsion effect on membrane–membrane apposition. The steric repulsion effect allows dynamic changes in cell interactions and thereby facilitates axon growth, branching and plasticity. Due to the nature of PSA biophysical properties, it is unlikely that PSA can act as a ligand or receptor for signalling molecules. However, it may be able to influence the signal transduction of other molecules indirectly by increasing intercellular space, by affecting the clustering of signal molecules, or by assisting the accumulation of growth factors. One such example is demonstrated by the influence of PSA on the interaction of NCAM and FGF receptor (FGFR). The firm adhesion mediated by unpolysialylated NCAM may prevent the interactions between NCAM and FGFR. The disruption of tight binding between NCAMs by PSA may assist the dimerization and clustering of FGFR and turn NCAM from an adhesion molecule to a signalling molecule (Kiselyov et al., 2005). PSA has also been shown to enhance or facilitate BDNF activation of its receptor TrkB and sensitize hippocampal pyramidal cells to the action of the growth factor (Muller et al., 2000). Administration of exogenous BDNF rescues newborn (P0) rat cortical neurons death. In addition, it also reversed the adverse effects on the P0 cortical neurons differentiation, induced by removal of PSA from cell surface in vitro (Vutskits et al., 2001). Several models have been proposed for the observed cross-talk between PSA-NCAM and BDNF (Vutskits et al., 2001). In a recent study, it was shown that NCAM and TrkB directly interacted via sequences in their intracellular domains and binding of TrkB to the NCAM regulates phosphorylation of NCAM and NCAM-dependent neurite outgrowth (Cassens et al., 2010).
**Fig. 1-4 The steric role of PSA at the cell surface.**
Schematic representation of molecular interactions during membrane-membrane contact. Note that while PSA is only attached to NCAM, its global effect on membrane-membrane apposition affects other contact-dependent receptors, such as cadherins. (The image is from Rutishauser, 2008).

1.6.2 ST8SiaIV (PST) and ST8SiaII (STX)
Polysialylation of NCAM was shown to be achieved by two α2,8-polysialyltransferases, ST8Sia IV (PST) and ST8Sia II (STX) (Cho and Troy, 1994; Nakayama et al., 1998; Franceschini et al., 2001). During development both enzymes are often expressed in the same region of the brain and work cooperatively on polysialylation. Both PST and STX transcripts are barely detectable at E8 and increased after E9 in mouse. STX is the dominating enzyme in the embryonic and early postnatal mouse, whereas PST prevails in the adult (Hildebrandt et al., 1998a; Ong et al., 1998). After birth the level of STX transcripts declines dramatically, whereas the level of PST transcripts gradually declines and maintains at a stable expression level. In adult nervous system, PST is more abundant than STX, and both transcripts are detectable only in restricted areas such as hippocampus, hypothalamus, and olfactory bulb where PSA expression persists (Seki and Arai, 1993; Angata et al., 1997; Phillips et al., 1997; Hildebrandt et al., 1998b; Hildebrandt et al., 1998a; Ong et al., 1998). The genetic ablation of PST generated mice in which the PSA synthesis was not significantly altered during development but almost completely absent in the adult (Eckhardt et al., 2000). In contrast, depletion of STX generated animals with ongoing PSA synthesis in the adult brain (Angata et al., 2004).
Both strains did not show gross anatomical abnormalities. Although distinct deficits have been identified in histological, electrophysiological, behavioural analyses (Eckhardt et al., 2000; Angata et al., 2004), the data suggest that each gene partially compensates for the absence of the other. However, STX and PST double knockout mice exhibit postnatal lethality and morphological brain anomalies, such as hydrocephalus, reduction in size of the internal capsule, and malformation of the anterior commissure and corticospinal tract (Weinhold et al., 2005). Furthermore, these phenotypes were not observed in mice deficient in NCAM, STX, or PST alone while many of these phenotypes were rescued in mice lacking NCAM, STX, and PST (Weinhold et al., 2005), indicating that NCAM protein in PSA deficient mice is responsible for the severe phenotypes seen in STX and PST double knockout mice.

It has been reported that, the degree of polysialylation by STX is lower than that by PST (about 20 residues less) (Angata et al., 2002). Both PST and STX are moderately related to another α2,8-sialyltransferase, ST8Sia III. However, PST and STX catalyze polysialylation of NCAM much more efficiently than ST8Sia III (Angata et al., 2000).

It remains unclear whether the biofunction of polysialylation formed by STX or PST is different. In one experiment, it has been shown both STX and PST can polysialylate NCAM-140 and polysialyated NCAM-140 facilitates neurite outgrowth better than nonpolysialylated NCAM-140. However, NCAM-140 polysialylated by PST served as a better substrate for neurite outgrowth than NCAM-140 polysialylated by STX (Angata et al., 1997).

1.6.3 Expression of PSA in the central nervous system

PSA is a developmentally regulated carbohydrate and is found to be most prevalent in the late development stage. The broadest expression of PSA within any tissue, including the nervous system, occurs in the early phases of its development, when it has been demonstrated to create permissive conditions for post-mitotic migration of neural and non-neural precursor cells. The classic example of the role of PSA in the neonatal nervous system has been in the rostral migration of large numbers of progenitors cells from the their birth place in the SVZ along a pathway to the olfactory bulb, where they become interneurons. Impaired migration of precursor cells has been reported in the
NCAM knockout mouse (Tomasiewicz et al., 1993) and PST/STX knockout mouse (Angata et al., 2007), or after removing or blocking PSA in diverse models (Ono et al., 1994; Hu et al., 1996; Kiss, 1998; Yoshida et al., 1999; Decker et al., 2000), indicating that polysialylated NCAM plays an important role in the mobility of precursor cells. In most cases, PSA is permanently lost after the migration process is completed. However, a subset of those cells, in particular neurons that produce long axonal tracts, retain PSA which allows their growing processes to bundle and branch appropriately during axon path finding (Tang et al., 1992). As with cell migration, the arrival of axons at their final destination is often accompanied by a loss of PSA, possibly to facilitate the establishment of stable synapses (Bruses et al., 1995; Bruses et al., 2002). In addition to the role of PSA in migration of neuronal precursor cells, it has now been recognized that the presence of PSA on newly generated cells in the SVZ can also influence the timing of their differentiation (Seki et al., 2007). Previous studies have demonstrated that PSA-NCAM acts as a negative regulator of myelination and that axonal expression of PSA is down-regulated to allow myelin deposition on PSA-free axons (Charles et al., 2000; Jakovcevski et al., 2007). In line with this, the time window identified for the major loss of PSA coincides with the onset of myelination (Uzman and Rumley, 1958; Matthieu et al., 1973). Inverse to PSA down-regulation, massive up-regulation of NCAM-120, the characteristic isoform of oligodendrocytes and myelin sheaths (Bhat and Silberberg, 1986, 1988), was observed. The majority of brain NCAM-120 was found in the PSA-free form, which is in agreement with the finding that migrating oligodendrocyte precursor cells but not mature myelinating oligodendrocytes express PSA (Trotter et al., 1989; Ben-Hur et al., 1998).

While overall PSA levels are greatly reduced in the adult brain, high levels of PSA persist in distinct regions that exhibit physiological plasticity, including regions of the hypothalamus, hippocampal, thalamus, habenular nuclei, mesencephalic central gray, lateral geniculate nucleus, and dorsal spinal laminae (Seki and Arai, 1991a, b; Bonfanti et al., 1992). Several studies (Theodosis et al., 1991; Nothias et al., 1997; Theodosis et al., 1999; Monlezun et al., 2005) have revealed that perturbation of PSA levels influences a wide range of CNS functions, and/or that PSA is associated with cellular elements known to be directly involved in behavioural plasticity, such as learning and memory, regulation
of circadian rhythms, secretion of hypophyseal hormones, chronic pain and emotional reactions such as anxiety and aggression (Schauer, 2009). Because of the anatomical and functional complexity of these regions, it has not been possible to define the role of PSA to the same degree described for developing systems.

Moreover, PSA is involved in the survival of neurons at development stage. Severe defects in anatomical organization of the forebrain associated with apoptotic cell death were reported in STX and PST double mutants (Angata et al., 2007). *In vitro* loss or inactivation of the PSA from NCAM on rat cortical neurons led to reduced differentiation and survival (Vutskits et al., 2001). An *in vitro* study showed that PSA is required for the survival of newly generated neurons (Vutskits et al., 2006; Gascon et al., 2007). By comparing cell death in NCAM knockout mice and wild-type animals and testing the effect of removing or blocking PSA-NCAM in a culture model, the results demonstrate that PSA-NCAM promotes neuronal survival by regulating p75NTR expression. It has been speculated that by limiting p75NTR expression, PSA-NCAM may protect newborn neurons from being dependent on trophic support before integration into olfactory circuits. This may ensure that enough cells arrive and compete in the olfactory bulb. Neurons having reached their appropriate place in the granule/glomerular layer, and having established synaptic connections, would be coupled to network activity that is crucial for their long-term survival (Miwa and Storm, 2005). The progressive down-regulation of PSA-NCAM and the increase in p75NTR expression during maturation would contribute to the elimination of non-integrated and/or misplaced cells.

### 1.6.4 Use of PSA in repair of central nervous system

Expression of PSA leads to reduction in cell–cell adhesion that can create permissive conditions for structural remodeling, an essential step towards CNS repair. One of the environmental factors that are consistently found to be negative for axon regrowth is the astrocytes that form a scar at the lesion site. It has been found that, the few axon sprouts that display a more persistent growth *in vivo* tend to be found in close apposition with a subpopulation of astrocytes that transiently upregulate PSA following lesion, and enzymatic removal of this PSA blocks the sprouting (Aubert et al., 1998; Dusart et al., 1999; Camand et al., 2004). These studies suggested that a more sustained elevation of
PSA might allow further regeneration to occur. Transduction of scar astrocytes with a construct that expresses PST has been used to obtain sustained high levels of PSA. With this treatment, a substantial portion of severed corticospinal tract axons as well as sensory ascending axons were able to grow through the spinal injury site (Zhang et al., 2007c). Similarly, it was found that induced PSA expression in a path extending from the SVZ to a lesion near the cortical surface increased recruitment of BrdU+/nestin+ cells along the path and into the injury site (El Maarouf et al., 2006). These displaced precursors were able to differentiate in a regionally appropriate manner. These findings suggest that induced PSA expression can be used as a strategy for promoting tissue repair involving both replacement of cells and rebuilding of neural connections. In the past few years, our group has generated LV carrying a PST cDNA (LV/PST) or a fused PST-GFP cDNA (LV/PST-GFP). When SCs transduced by LV/PST were transplanted into the cerebellum of L1/GAP-43 transgenic mice, it was shown that much more Purkinje cell axons regenerating into the transplant than the control group (GFP/SCs) (Zhang et al., 2007a). When LV/PST was injected into the dorsal root entry zone (DREZ), it transduced the cells in spinal cord and promoted a significant number of injured sensory axons to cross DREZ into spinal cord (Zhang et al., 2007b). It was also observed that a large number of SCs migrated into the PSA-expressing region of the spinal cord and many regenerating axons were closely associated with the SCs. Similarly, engineered expression of PSA in combination with a conditioning lesion in spinal cord has been shown to promote the regeneration of sensory axons through the injury site (Zhang et al., 2007c). The results indicate that over-expression of PSA on grafted SCs or on cells in spinal cord can promote axon regeneration. More recently, it was found subdural infusion of PSA mimetic over 2 weeks immediately after spinal cord compression injury in mice, animals showed improved functional recovery assessed by locomotor rating and video-based motion analysis over a 6-week observation period. Moreover, compared with control mice, higher numbers of cholinergic and glutamatergic terminals and monaminergic axons in the lumbar spinal cord, and better axonal myelination were found in PSA mimetic-treated mice (Mehanna et al., 2010).

In addition of directly inducing the expression of PSA in the host tissues or delivery PSA mimetic, over-expression of PSA on grafted SCs also showed promising
effects on promoting axonal growth and neural repair. When SCs transduced by LV/PST were transplanted into the cerebellum of L1/GAP-43 transgenic mice, it was shown that much more Purkinje cell axons regenerating into the transplant than the control group (GFP/SCs) (Zhang et al., 2007a). Moreover, when SCs transduced by LV or retroviral vector encoding STX were transplanted into compression injury iste of mice spinal cord (Papastefanaki et al., 2007) or lysophosphatidylcholine induced demyelinated mice spinal cord (Bachelin et al., 2010), they showed enhanced migration ability towards lesion site (Bachelin et al., 2010) and remyelination potential (Papastefanaki et al., 2007; Bachelin et al., 2010). Improved locomotor recovery was also observed (Papastefanaki et al., 2007).

It has been suggested that PSA facilitate cell migration, neurite outgrowth and synaptic plasticity because its hydration volume could enhance flexibility of cell interactions. Evidence for receptors of PSA has so far been elusive. In a recent study, histone H1 was dentified as binding partner of PSA. Administration of histone H1 to lesioned femoral nerves promotes survival of motoneurons, axonal regrowth, precision of reinnervation, and better regeneration of the injured femoral nerve, leading to a remarkable complete functional recovery of locomotion after injury (Mishra et al., 2010) which is similar to those positive effects on recovery after femoral nerve lesion in mice treated with PSA-mimicking peptides (Mehanna et al., 2009). More studies need to be carried out to investigate the interaction between histone H1 and PSA.

The neuroprotective effect of PSA-NCAM could also be important under pathological conditions. PSA is known to be reexpressed in lesion contexts, such as in mechanical damage, focal ischaemia, axotomy, stroke and epileptic seizures (Gascon et al., 2007). Remarkably, blockade or enzymatic disruption of PSA leads to an impaired repair process (Daniloff et al., 1986; Bonfanti et al., 1996) and the NCAM knockout mice exhibit deficient recovery after cortical lesions (Troncoso et al., 2004). It has been reported that, in a mice model of ALS, surviving motoneurons in transgenic animals express PSA-NCAM (Warita et al., 2001), which raises the possibility that PSA re-expression protects undamaged and functional neurons from cell death. Furthermore, it was found that enzymatic removal of PSA resulted in increased hippocampal neurons death via glutamate-induced excitotoxicity (Hammond et al., 2006) which indicates PSA could prevent excitotoxicity via inhibition of NR2B subunit-containing NMDA receptors.
1.7 Hypotheses and aims

As discussed above, to make SC transplantation a more clinically applicable procedure, genetic modification of SCs in vitro before transplantation is an option to overcome the drawbacks of SCs in CNS repair. In this study, we genetically modified SCs by LV-mediated expression of PST to over-express PSA.

SCs normally express little or no PSA. It is speculated that over-expression of PSA on SCs will reduce their adhesion and facilitate their migration. Engineered expression of PSA on SCs has been shown to enhance their migratory potential in vitro (Lavdas et al., 2006). As we have shown that engineered expression of PSA on spinal cells can promote sensory axons growing across DREZ and facilitate the migration of endogenous SCs into the spinal cord (Zhang et al., 2007c), we also speculate that over-expression of PSA on spinal cells will provide a pathway to facilitate the migration of transplanted SCs. Recently, it was reported that PSA-expressing SCs showed enhanced migratory potential without impairment of their myelinating ability in vitro (Lavdas et al., 2006). It is reasonable to speculate that over-expression of PSA on grafted SCs can enhance their ability to provide a supportive environment for regrowth and remyelination of damaged axons.

Extensive SC death after transplantation is another issue need to be solved. PSA has been shown to promote neuronal survival in physical and pathological conditions, although the underlying mechanisms for such phenomena are unclear. We propose that modification of SCs to over-express PSA may improve the survival rate of grafted SCs.

In this study we studied over-expression of PSA on SCs on promoting their survival after transplantation, their migration in uninjured and injured spinal cord, their integration with the host tissue and their interactions with astrocytes in vitro.
Chapter 2

Materials and methods

2.1 Production of lentiviral vectors

2.1.1 Preparation of lentiviral transfer vector

The mouse PST cDNA was kindly provided by M. Eckhardt (Eckhardt et al., 1995) and subcloned into the lentiviral transfer vector pRRL (Naldini et al., 1996). In order to directly visualize the expression and localization of PST, PST cDNA was fused to the N-terminal of GFP cDNA to generate PST-GFP (Zhang et al., 2007c). The addition of GFP to the C-terminal of PST does not interfere with its enzymatic activity (Angata et al., 2001). mCherry (a kind gift from Roger Tsien) and GFP cDNAs were also subcloned into pRRL transfer vectors.

Transfer vectors were transformed into NovaBlue competent cells (Novagen) and transformants were selected on LB agar plates containing ampicillin (100 µg/ml). After overnight incubation at 37°C, colonies were picked and grown in 3 ml Terrific Broth medium supplemented with 100 µg/ml ampicillin overnight until the ideal density was reached. Plasmid DNAs were then isolated and purified using Miniprep kit (QIAprep). To check whether the mini-prep plasmids have the predicted sequence, plasmid DNAs were digested with selected restriction enzymes in 20 µl at 37°C for 1 h. The digested plasmids were loaded into an 1% agarose gel to identify the sizes of the DNA bands. After confirmation, the midi-cultures were grown, followed by midi-prep (QIAprep kit) to produce larger quantity of plasmids for transfection. Plasmid DNA concentration were measured using an UV spectrophotometre, with absorption wave length at 260 nm. The purity of the DNA preparations was estimated by calculating the ratio of the absorbance at 260 nm and 280 nm. It should be between 1.65 and 1.85. Plasmid DNAs were diluted to 1 µg/µl. 0.5 µg of each plasmid was digested again with restriction enzymes to further confirm the DNA sequences and the concentrations of DNAs. Plasmid DNAs were stored at -80°C until use.

2.1.2 Production of lentiviral vectors

The self-inactivating LV system was obtained from Prof. J.Verhaagen in Netherlands
Institute for Brain Research with the permission from Prof. Naldini in University of Naples Federico II, Italy. The production of the LV carrying the fused mouse PST-GFP (LV/PST-GFP) or LV carrying GFP (LV/GFP from J.Verhaagen) or LV carrying mCherry (LV/mCherry) was based on the protocol of Dull et al. (Dull et al., 1998).

HEK293T cells (2.5-5 × 10^6) were plated in a 10 cm dish in HEK cell complete medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS, Invitrogen), 100 unit/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 2 mM L-Glutamine (Sigma-Aldrich). Cells were cultured in a humidified incubator at 37°C with 5% CO₂ + 95% air. When the required number of dishes of HEK cells was reached and the cell density was about 90% confluency, transfection was carried out using calcium precipitation method. Medium in the dishes was replaced 2 h before transfection to ensure the pH in the medium was 7.2 as pH will affect the transfectin efficiency. The amount of transfection mix prepared was based on the numbers of 10 cm dishes of cells (see the table below). Varied amount of sterile H₂O, 0.1 × Tris-EDTA (TE), transfer vector, package vector (PACK), and envelope vector (ENV) were mixed together (Table. 2-1). CaCl₂ was then added and mixed again. To obtain high transfection efficiency, the mixture was vortexed vigorously while adding equal volume of HEPES-buffered saline (HBS) drop by drop. The speed was about at 2 drops per second. The mixture was then immediately added to the dish. The dishes were swirled gently in different directions to make sure the transfection mixture was distributed evenly.
Table 2-1 Preparation of transfection mixture

<table>
<thead>
<tr>
<th>Components</th>
<th>For 1 × 10 cm dish</th>
<th>For 2 × 10 cm dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile H₂O</td>
<td>180 μl</td>
<td>360 μl</td>
</tr>
<tr>
<td>0.1 x TE</td>
<td>250 μl</td>
<td>500 μl</td>
</tr>
<tr>
<td>ENV*</td>
<td>3.5 μl</td>
<td>7 μl</td>
</tr>
<tr>
<td>PACK*</td>
<td>6.5 μl</td>
<td>13 μl</td>
</tr>
<tr>
<td>Transfer vector*</td>
<td>10 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>2.5 M CaCl₂</td>
<td>50 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>2 x HBS</td>
<td>500 μl</td>
<td>1,000 μl</td>
</tr>
<tr>
<td>Total</td>
<td>1,000 μl</td>
<td>2,000 μl</td>
</tr>
</tbody>
</table>

*All plasmids were diluted to 1 μg/μl.

The medium was replaced 14 to 16 h after transfection with 9 ml medium containing 2% FBS. Over 90% cells should be green at this time if the transfection was good. Medium was collected 48 h after transfection and centrifuged at 1000 rpm for 5 min to remove the cell debris. The medium was then filtered through 0.22-μm pore-sized cellulose acetate filters. For concentration of the viral vectors, the medium from three dishes was loaded into a 28 ml Ultracone tube and concentrated by centrifugation at 20,000 rpm (≈74,000 × g) for 2.5 h in a Sorvall Surespin 630 swing-bucket rotor. The supernatant was discarded carefully by suction. To remove the medium as much as possible, the tubes were then put upside down for 5-10 min. The medium around the neck of the tubes was removed by suction. The viral vector pellet was resuspended in 50 μl of filtration sterilized PBS and incubated at room temperature (RT) for 30 min to allow it disperse in PBS thoroughly. The resuspended viral particles were then aliquoted in screw-capped tubes (5 μl each) and stored at -80°C.

2.1.3 Titration of lentiviral stocks

The day before titration, HEK cells were plated at 2.5-5 × 10⁵ cells/well in 6-well-plates in HEK cell complete medium. Serial dilutions of viral stock from 10⁻³ to 10⁻⁸ were prepared in HEK cell complete medium (Fig. 2-1). Culture medium was replaced with 1 ml fresh medium plus 1 ml diluted viral stock. Twenty four h after infection, medium was replaced
with fresh one and the cells were incubated for another 24 h. Cells were then fixed and the transduced cells expressing GFP or mCherry were counted for calculating the transduction unit (TU) according to the formula below:

Number of green (or red for mCherry) cells (in one field of 10× objective lens) × viral stock dilution × 700 = TU/ml

The titre for LV/PST-GFP was in the range of 0.5 - 1 × 10^{10} TU/ml, and 1 - 7 × 10^{10} TU/ml for LV/GFP and LV/mCherry.

Fig. 2-1 Preparation of viral serial dilutions for titre evalation
Serial viral stock dilutions (from 10^{-3} to 10^{-8}) were made in HEK cell complete medium.
Comp. Medium = HEK cell complete medium.

2.2 Cell culture and transduction

2.2.1 HeLa-NCAM140 cell lines

2.2.1.1 Thawing frozen cell stocks
HeLa-NCAM140 stable cell lines were kindly provided by Minoru Fukuda (Angata et al., 1997). A vial of frozen cells was taken out from liquid nitrogen and kept on dry ice. The cells were incubated in water bath at 37°C under gently shaking until completely thawed. The cells were transferred into a 15 ml centrifuge tube containing 10 ml pre-warmed complete medium (same as HEK cell complete medium). Cells were then centrifuged at 1,000 rpm for 5 min and the supernatant was carefully removed. Cells were resuspended in
1 ml complete medium by pipetting up and down and transferred into a 10 cm Petri dish. The medium was replaced with fresh one after 12-24 h.

2.2.1.2 Subculturing

Cells were normally confluent and ready for passaging by the second or the third day after thawing and then every 3-4 days afterwards, at a dilution of 1 to 4 or 1 to 6 depending on the density of the cells. The medium was removed and cells were washed with Hanks’ balanced salt solution (Sigma-Aldrich). Two ml of 0.25% trypsin/EDTA solution (Sigma-Aldrich) was added and cells were incubated at 37°C for several min until they began to detach. Eight ml complete medium was added to neutralize trypsin. Cells were tritutrated several times and transferred into a 15 ml centrifuge tube, then centrifuged at 800 rpm for 5 min. After removing the supernatant, the cell pellet was resuspended in 1 ml fresh complete medium. The density of cells was determined by haemocytometry. The number of cells seeded into a new Petri dish depended on the size of the Petri dish. Fresh medium was added to reach the desired volume.

2.2.1.3 Transfection of PST into HeLa-NCAM140 cells

HeLa-NCAM140 cells were transfected with pRRL/PST-GFP using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) with slight modification. Cells were grown on poly-L-lysine (PLL, Sigma, 10 μg/ml) coated coverslips placed in a 35 mm dish. To obtain high transfection efficiency, transfection was carried out when the cells reached 90-95% confluency. To prevent cell death, growth medium was replaced with the medium without antibiotics before transfection. The amounts of Lipofectamine 2000, DNA and medium used for transfection were adjusted according to the size of Petri dish and cell types (Table. 2-2).
### Table. 2-2 Preparations of transfection of Lipofectamine 2000

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Volume of Plating Medium</th>
<th>DNA (µg) and Dilution Volume (µl)</th>
<th>Lipofectamine 2000 (µl) and Dilution Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 well</td>
<td>500 µl</td>
<td>1 µg in 50 µl</td>
<td>3 µl in 50 µl</td>
</tr>
<tr>
<td>35 mm</td>
<td>2 ml</td>
<td>2 µg in 100 µl</td>
<td>6 µl in 100 µl</td>
</tr>
<tr>
<td>60 mm</td>
<td>5 ml</td>
<td>4 µg in 250 µl</td>
<td>12 µl in 250 µl</td>
</tr>
<tr>
<td>10 cm</td>
<td>10 ml</td>
<td>10 µg in 1 ml</td>
<td>30 µl in 1 ml</td>
</tr>
</tbody>
</table>

Two 1.5 ml tubes were prepared for each transfection. The required amounts of Opti-MEM I medium (Invitrogen) were dispensed into each tube. Lipofectamine 2000 reagent was diluted into one of the Eppendorf tubes and incubated for 5 min at RT. The required quantity of plasmids was added into the second Eppendorf tube. The diluted Lipofectamine 2000 was then combined with the diluted plasmid by mixing them gently and incubated for 20 min at RT. This stage allows the DNA-Lipofectamine 2000 complexes to form. The complexes were added directly to each dish and mixed gently by rocking the dish. The cells were incubated at 37°C in a humidified incubator with 5% CO₂. Medium was replaced with complete medium after 12 h to minimize the toxicity caused by Lipofectamine 2000.

#### 2.2.1.4 Detection of PSA expression on HeLa-NCAM140 cells

Forty-eight h after transfection, some PST transfected cells on coverslips were fixed with cold 4% paraformaldehyde (PFA) for 10 min, washed in PBS 3 × 5 min and then processed by immunocytochemistry to identify PSA expression. It was found GFP fluorescence mainly distributed in the Golgi apparatus in pRRL/PST-GFP transfected cells where PST located.
Fig. 2-2 Expression of PSA on Hela-NCAM140 cells
HeLa-NACM transfected with pRRL/PST-GFP immunostained with anti-PSA antibody mAb735 (red in B, C). Arrows point to PST-GFP fusion protein. Scale bar = 50 µm.

2.2.2 HEK cells
HEK 293T cells were cultured with the similar procedures as for HeLa-NCAM140 cells.

2.2.3 Schwann cell culture and transduction
2.2.3.1 Purification and culture of Schwann cells
SCs were purified from 2-3 days old (P2-3) neonatal Wistar rats according to the method of Brockes et al. (Brockes et al., 1979). The dissecting area and equipment were sterilized with 70% ethanol. A corkboard was covered with tin foil and then sterilized with 70% ethanol. P2-3 neonatal rats were killed by decapitation. The bodies were pinned onto the corkboard and sterilized with 70% ethanol. Sciatic nerves and nerves from brachial
plexus were dissected out under a dissection microscope, and placed in a 35 mm dish containing L15 medium (Sigma-Aldrich), which was kept on ice. After being stripped of epineural sheath and connective tissue, the desheathed nerves were placed in a fresh dish containing L15. Nerves were incubated with 2.5% trypsin (Sigma-Aldrich) (100 µl per pup) and equal volume of 0.4% collagenase (Type II, Worthington Biochemical Corporation) at 37°C for 35 min. Nerves were dissociated by mechanical trituration using a 1 ml tip and incubated for a further 10 min. They were then trituated again with a 200 µl tip until they were fully dissociated. Equal volume of fresh complete medium was added to stop the enzymatic digestion. The digested nerves were then passed through a filter (70 µm in pore-size, BD), transferred to a 15 ml tube and centrifuged for 10 min at 1,000 rpm. The supernatant was carefully removed. Cells were resuspended in fresh SC culture medium (DMEM medium containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml β-hergulin (R&D Systems), 2 µM forskolin (Sigma), 25 ng/ml FGF (PeproTech) and 5 µg/ml insulin (Sigma) and plated in a PLL (Sigma, 10 µg/ml) coated 10 cm dish. Culture medium was replaced every 3 days afterwards. SCs were identified with immunostaining using anti-p75NTR or anti-S100 antibodies.

2.2.3.2 Transduction of Schwann cells
Cultured SCs were transduced with either LV/GFP (referred as GFP/SCs) or LV/PST-GFP (referred as PST/SCs). Due to weak GFP fluorescence from PST-GFP fusion protein expressed in SCs in vivo, LV/PST-GFP transduced SCs were co-transduced with LV/GFP for easy identification. To further confirm the polysialylation of NCAM, PST/SCs or naïve SCs were washed in pre-chilled Hanks’ balanced salt solution and harvested by a cell culture scraper without TE treatment to avoid membrane protein degradation. The detached cells were transferred into a 15 ml centrifuge tube, spun at 800 rpm for 5 min, followed with PBS washing, and pelleted by centrifugation again. Cells were either used immediately or stored at -80°C for Western blotting analysis. Some samples were treated with 0.7 units endoneuraminidase (Endo-N, AbCys S.A, France) for 1 h at 37°C to remove PSA from NCAM.

2.2.3.3 Preparing LV-transduced Schwann cells for transplantation
PST/SCs or GFP/SCs were washed with 1 ml of DMEM before adding 600 µl of 0.25% trypsin/EDTA to dissociate the cells. The cells were checked under a microscope to make sure most of the cells were detached from the dishes before 2 ml of complete medium was added to neutralize trypsin. Dissociated cells were then transferred into a 15 ml centrifuge tube. One ml complete medium was added to the dish to collect the cells remained in the dishes. Cells were then centrifuged at 900 rpm for 10 min. After removing the medium the cell pellet was resuspended in 1 ml of DMEM and transferred to a 1.5 ml tube. The resuspended cells were centrifuged at 2,500 rpm for 5 min. The wash step was repeated once more. Cell pellet was resuspended in 5-10 µl DMEM. Cells were counted and the density of cells was adjusted to 100,000 cells/µl. Cells were kept on ice and ready for transplantation.

2.2.4 Astrocyte culture and transduction

Astrocytes were purified from the cerebral cortex of neonatal (P2-3) rat according to McCarthy and de Vellis (Braunewell et al., 1995). Two P3 neonatal pups of Wistar rat were sacrificed by decapitation. Using micro-dissection scissors, the skin was opened at the midline of the head, cutting from the base of the skull to the mid-eye area. After folding back the skin flaps with the scissors, the skull was cut at the midline fissure, without cutting into the brain tissue. The raised skullcap was removed with the curved forceps, applying slight pressure. The brain was then released from the skull cavity by running a micro-spatula underneath and along the length of the brain from the olfactory lobes to the beginning of the spinal cord.

Dissected brains were gently transferred to a 35 mm Petri dish containing 2.5 ml DMEM/F12 serum free medium (SFM). One brain was removed from the 35 mm Petri dish to the inverted lid of a 35 mm Petri dish. Cerebral hemispheres were removed using microdissecting forceps and the olfactory lobes were no longer attached. The cerebral hemispheres were immediately immersed in SFM in another sterile 35 mm Petri dish. Hemispheres were then placed onto the inverted lid of a second Petri dish. Meninges and blood vessels were removed using microdissecting forceps. The hemisphere piece was placed into a fresh Petri dish containing DMEM/F12 SFM. The brain was chopped and incubated with 0.1% trypsin in DMEM for 30 min at 37°C in a humidified incubator with
5% CO$_2$. The mixture was then triturated in triturating solution (10% FBS in DMEM containing 100 μg/ml soybean trypsin inhibitor, 0.5 μg/ml DNase I) and the cells were centrifuged and resuspended in DMEM + 10% FBS. Cells were plated in PLL-coated (10 μg/ml) 75 cm$^2$ tissue culture flasks at a concentration of $1.5 \times 10^7$ cells in 10 ml of medium or in 25 cm$^2$ tissue culture flasks at a concentration of $5 \times 10^6$ cells in 5 ml of medium. Incubating the flasks with lid loosened at 37°C in a moisturized incubator with 5% CO$_2$ and 95% air, allowing the cells sufficient time to adhere and begin multiplying. The medium was changed every 48-72 h afterwards. After 6-7 days' culture, the medium was changed to remove microglia which were floating in the medium. At the end of 7-9 days phase-dark, process-bearing cells were observed to approximate a confluent phase-gray bed layer of cells. The culture medium was then changed again. Flasks were closed tight with lids, placed on a shaking platform in a horizontal position, and were shaken at 200 rpm for 6 h at 37°C to separate the oligodendrocytes from the astrocytes. Medium was changed and the flasks were shaken for 18 h (the discarded medium usually contains oligodendrocytes, dividing astrocytes and macrophages). The medium was changed again and the shaking process was repeated for further 24-48 h to remove additional oligodendrocytes. The flasks were then replenished with fresh culture medium and shaken at 100 rpm, until fewer than 10 phases–dark cells per microscope field of view (10× objective lens) were observed. Fresh medium was replenished every 2 days afterwards. According to the experimental design, some cultured astrocytes were transduced with LV/PST-GFP or LV/GFP. Five days later cells were subsequently subcultured. All medium was removed from the flasks, and the cells were firstly washed with 5 ml of Hanks’ balanced salt solution and then incubated with 2 ml of 0.25% trypsin/EDTA solution at 37°C for 5-10 min until the cells were completely disaggregated. The cells were resuspended in 8 ml DMEM plus 10% FBS and transferred to a 15 ml centrifuge tube. After being centrifuged for 5 min at 800 rpm, the medium was discarded and cells were resuspended in 2 ml fresh culture medium. The cell density was determined by haemocytometre and $4 \times 10^5$ cells were plated in each 35 mm Petri dish or on coverslips in Petri dishes depending on the purpose of each individual experiment.

2.2.5 Neural stem/progenitor cell culture
NSCs/NPCs were obtained from a colleague in our Centre. The procedure for isolation and proliferation of neural stem/progenitor cells was performed according to the protocols of Pacey *et al.* (Pacey, 2006) and Hutton and Pevny (Hutton, 2008). P7 neonatal C57BL/6J mice were sacrificed and sprayed with 70% ethanol. Under sterile conditions, the brain tissue of the mice was isolated and washed with PBS for three times, then cut into small pieces. Brain tissue clumps were treated with 0.05% trypsin (Gibco) at 37°C for 5 min, and filtered with a 70 µm cell strainer (BD). The cells were collected and centrifuged at 1000 rpm for 7 min. The precipitated cells were resuspended and seeded into a 60 mm dish in the serum-free medium containing DMEM/F12 (Invitrogen), B27 (2%, Invitrogen), bFGF (20 ng/ml, Invitrogen) and epidermal growth factor (EGF, 20 ng/mL, R&D). The cells were cultured at 37°C with 5% CO₂. Medium was replaced every day. After 6–7 days of suspension culture, the neurospheres were re-plated onto matrigel-coated 60 mm tissue culture dishes for adherent culture in the same medium for another 2–3 days. The adherent neurospheres were dissociated with 0.25% trypsin/EDTA into single cells and passaged for monolayer culture. The NPCs in mono-layer culture for 2–3 passages were characterized by immunostaining with anti-nestin antibody.

### 2.3 Surgeries

#### 2.3.1 Cell transplantation and spinal cord crush injury

All the surgery procedures were performed strictly in accordance with UK government legislation on animal care and genetic manipulation. Adult female Wistar rats (200-250 g) were used for SC transplantation experiments.

*Transplantation of Schwann cells to intact spinal cord*

Animals were deeply anaesthetized with isoflurane. A laminectomy was performed to expose spinal cord at T8 level. One µl containing 100,000 PST/SCs or GFP/SCs was injected into the dorsal column (1.2 mm depth from dura) using a Hamilton syringe connected to a 33 gauge fine metal needle. The injecting speed was 200 nl/min controlled by UltraMicroPump II (World Precision Instruments, Sarasota, FL, USA). The needle was left at the injection site for a further 5 min to prevent leakage from the needle track. Animals were treated with or without ciclosporin (10 mg/kg intraperitoneally, daily) to
assess the effect of immune response on the survival of transplanted SCs. The rats were sacrificed by perfusion with 4% PFA at 1, 7, 19, and 30 days after transplantation. The segments of spinal cords containing the transplanted SCs were removed.

**Transplantation of Schwann cells to the spinal cord caudal to the lesion site**

Spinal cord at T8 level was crushed with a pair of fine forceps at a depth of 1.5 mm for 5 s. One μl (100,000 cells) of PST/SCs or GFP/SCs were injected into the dorsal column 2.5 mm caudal to the lesion site as described above. To study whether engineered expression of PSA in the spinal cord can further enhance the migration of transplanted SCs towards the lesion sites, LV/mCherry (as control) or LV/PST-GFP were injected adjacent to the lesion sites immediately after transplantation of PST/SCs or GFP/SCs. The muscle and skin incisions were closed with sutures. Animals were treated with ciclosporin daily. Three weeks after transplantation animals were killed by perfusion with 4% PFA and the segments of spinal cord containing the injury site and transplanted SCs were removed. Animals were monitored daily after surgery. Weight of animals was recorded daily. Saline was administered for animals with severe weight loss, up to 20 ml/day. Bladder expression was performed one or two times per day, until the animal recovered its bladder function.

2.3.2 Tissue preparation

Animals at different time points after surgery were briefly anaesthetised by CO₂ and then overdosed with Euthanal (60 mg/kg) by intraperitoneal injection. When reflexes were no longer present but before respiration failed, the ventral abdominal wall was opened along the midline and the diaphragm was cut around part of its periphery. The rib cage was opened with scissors along both sides and pulled back and secured with clamps to expose the heart widely. The right atrium was opened with a pair of scissors to release venous blood. A blunt cannula was immediately inserted into the left ventricle and up into the aorta. The animals were first exsanguinated with 200 ml of 0.9% saline and then perfused with 300 ml of pre-chilled 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords containing the transplanted SCs and the lesion site were removed and postfixed in the same fixative overnight and then cryoprotected with 30% sucrose in 0.1 M PB at 4°C.
until they had sunk. Samples were then embedded with OCT on dry ice. Segments of T8-9 spinal cords were cut longitudinally at a thickness of 15 µm in a cryostat. The sections were collected on polysine-coated slides (BDH).

2.4 Immunoblotting

2.4.1 Sample preparation

2.4.1.1 Protein dissociation from cultured cells

The collected cell pellets were resuspended in cell solubilization buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.8% NP-40, pH 7.4) containing Complete Mini protease inhibitors (Roche, 1 tablet added into 7 ml solubilization buffer) at a 1:5 weight/volume ratio and kept on ice for 1 h with occasional tapping of the tubes. They were then centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were collected, aliquoted, and used for sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) immediately or stored at -80°C.

2.4.1.2 Measurement of protein concentration

Concentrations of solubilized proteins were measured using the DC Protein Assay kit from Bio-Rad. A standard curve was constructed using bovine serum albumin (BSA, New England Biolab) diluted with the same solubilization buffer for the sample preparation in the following concentrations (mg/ml): 0.2, 0.4, 0.7, 1.0 and 1.5. Five µl of standards and samples were added into Eppendorf tubes. For the blank, 5 µl of solubilization buffer was used. Twenty-five µl of Reagent A and 200 µl of Reagent B were added into each tube. The components in each tube were mixed gently by inverting the tube for several times. After incubation for 15 min, the absorbance was then read at the wavelength of 750 nm.

2.4.1.3 Endo-N treatment

Solubilized proteins (10 µg) from LV/PST-GFP transduced SCs were treated with 0.7 unit of Endo-N (AbCys S.A, Paris, France) at 37°C for 1 h to remove PSA from NCAM.

2.4.2 SDS-PAGE

2.4.2.1 Gel casting
Separating and stacking gel solutions were prepared separately (Table. 2-3). Usually, 10 ml separating and 3 ml stacking gel solutions are enough for 2 mini gels (10 cm × 10 cm).

Table. 2-3 SDS-PAGE preparation

<table>
<thead>
<tr>
<th>Components</th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
<td>8%</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.0 ml</td>
<td>4.63 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>3.3 ml</td>
<td>2.67 ml</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1.0 M Tris pH 6.8</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

*Ammonium persulphate (APS) was prepared freshly in water.

The ingredients of separating gel were mixed gently and the solution was poured quickly into gel cast until its height reached 1 cm below the bottom line of the comb. The gel was overlaid with distilled water very carefully to ensure a flat surface and to exclude air. After the gel polymerized completely (about 20-30 min), the distilled water was removed. The stacking gel solution was mixed well before being poured on top of separating gel and a comb was inserted. After the gel set (about 10-15 min), the comb was removed and the gel tank was filled with electrophoresis running buffer (25 mM Tris-base, 25 mM glycine, 0.1% SDS). Gel wells were washed with running buffer using a syringe and the air bubbles between the two glass-plates were removed before the samples were loaded.

2.4.2.2 Sample loading and electrophoresis

Samples were mixed with 5 × sample buffer (62.5 mM Tris-HCl, PH 6.8, 20% glycerol, 2% SDS, 0.025% Bromophenol blue, 5% β-mercaptoethanol) in the ratio of 4:1 and heated at
95°C in a heat block for 5 min to denaturize the proteins. Samples were then chilled on ice and ready for loading. Samples (10 µg proteins) and Precision Plus Protein Dual Color Standards from Bio-Rad were loaded into the wells of the polyacrylamide gel. The gels were run using a mini gel system (Bio-Rad) at 100-150 V constantly for 1-2 h until the markers appeared in the expected places.

2.4.2.3 Membrane transfer
Polyvinylidifluoride (PVDF) membrane (Bio-Rad) and filter paper were cut to size slightly bigger than the gel. PVDF membrane was soaked in methanol for 5 min. Gel was removed from electrophoresis apparatus, trimmed (the stacking gel was discarded) and rinsed in distilled water remove SDS. Pads, filter papers, gel and wet PVDF membrane were then pre-soaked in transfer buffer (120 mM glycine, 15.6 mM Tris-base, and 20% methanol) for 10 min. The “transfer sandwich” was assembled in the following order from cathode (-) to anode (+): pad, filter paper, gel, PVDF membrane, filter paper, pad. Caution was taken to avoid air bubbles during the sandwich preparation. The “transfer sandwich” was then placed in a mini gel transfer tank (Bio-Rad). An ice pack was placed in to cool down the buffer during transfer. The transfer tank was filled with 1 × pre-chilled transfer buffer (about 800 ml for one transfer) and the separated proteins were transferred at 200 mA constant at 4°C for 1 h.

2.4.2.4 Immunopробing
The membrane was removed from the transfer apparatus and washed in Tris buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 min twice on a platform shaker. The wash is important to reduce spotted or blotched background. The membrane was marked with pencil for the orientation at this stage. After blocking in 10% skimmed milk freshly made in Tris buffered saline (TBS) on a shaker for 1-2 h at room temperature or overnight at 4°C the membrane was rinsed in TBS with Tween 20 (TTBS, TBS + 0.1% Tween 20, Sigma-Aldrich) for 5-10 min. To minimize the usage of antibodies the membrane was trimmed to an appropriate size and probed with different primary antibodies in TTBS/10% skimmed milk overnight at 4°C or for 2 h at RT on a shaker. A mouse monoclonal antibody mAb735 (gift from Hildebrandt and Gerardy-Schahn in
University of Hanover, Germany, 1:1000) was used to immunoblot PSA. The membrane was washed $3 \times 20$ min in TTBS, followed by incubation with anti-mouse IgG-horseradish peroxidase (Amersham, 1:10,000) for 90 min at RT on a shaker. The excess secondary antibody was rinsed off with TTBS $3 \times 10$ min. The membrane was then ready for detection with ECL Plus Western Blotting Detection Reagents (Amersham).

### 2.4.2.5 Detection

Amersham ECL western blotting detection kit (GE healthcare) was used in this step. The detection reagents were equilibrated to RT before opening. The detection solutions A and B were mixed in a ratio of 40:1. The final volume of detection regent required was 0.1 ml/cm² of membrane. The excess wash buffer was drained from the membrane and the membrane was placed on a sheet of cling film with protein side facing up. The mixed detection reagent was pipetted on to the membrane evenly. The reagents should have covered the entire surface of the membrane. Membrane was incubated for 5 min at RT. The excess detection reagent was drained off. Membrane was placed protein side down to a piece of Saran Wrap and wrapped gently to remove any air bubble. The wrapped membrane was then placed protein side up in an X-ray film cassette. The following stages were carried out in a dark room. A sheet of Hyperfilm ECL (Amersham) was cut to an appropriate size and placed on top of the membrane. The cassette was closed and the film was exposed for different times in order to achieve the best signals and minimum of background. The exposure time varied from 5 s to 30 min.

### 2.5 Immunocytochemistry and Immunohistochemistry

#### 2.5.1 Immunocytochemistry

Cells were fixed in 4% PFA washed in PBS containing 0.2% Triton X-100, then blocked with 10% normal donkey serum (NDS) or 10% normal goat serum (NGS) in PBS according to the species of the secondary antibodies. The following primary antibodies were used: polyclonal anti-GFAP antibody or monoclonal anti-GFAP antibody, polyclonal anti-p75NTR antibody, monoclonal anti-PSA antibody, monoclonal anti-BrdU antibody, pan anti-CSPGs, CS-56. Primary antibodies were diluted with 10% NDS (or 10% NGS) and 1% BSA containing 0.2% Triton X-100 in PBS and applied for 60 min at
RT, followed by incubation with appropriate secondary antibodies for 60 min. To detect cell membrane proteins, no detergent was used in blocking solution and washing buffer. To detect other proteins, Triton X-100 or Tween 20 was used in blocking solution and washing buffer to permerbilize the cell membrane.

For nucleus staining, 4’,6’-diaminidino-2-phenylindole (DAPI, 0.0002%, Sigma-Aldrich) was applied for 5 min. After final wash, coverslips were mounted in 1:9 PBS-glycerol.

For BrdU staining, cells were fixed in 4% PFA, followed by treatment with 2 M HCl for 1 h. After thorough wash, cells were immunolabeled with anti-BrdU antibody overnight at 4°C, followed by incubation with TRITC conjugated anti-mouse IgG (1:600) for 60 min.

2.5.2 Immunohistochemistry

Routine immunohistochemistry was carried out on parasagittal cryosections (15 µm thick) of spinal cords. For GFAP/PSA or CSPGs/PSA double staining, sections were first incubated with blocking solution (10% NDS or NGS) for 2 h, and then in a mixture of antibodies against GFAP or CSPG and PSA diluted in blocking solution overnight at 4°C. After thorough wash with PBS (3 × 10 min), the sections were subsequently incubated with a mixture of appropriate secondary antibodies for 2 h. The sections were washed in PBS, mounted in 1:9 PBS-glycerol.

To detect myelination of implanted SCs, mouse monoclonal anti-P0 antibody was used, slides were rinsed with PBS (3 × 5 min), pre-treated with ice-cold methanol for 5 min at -20°C followed by washing with PBS (3 × 10 min). Then an appropriate secondary antibody was applied.

The details of the antibodies used in this thesis for immunostaining are shown in Table. 2-4
Table. 2-4 Details of antibodies used

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
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</tr>
<tr>
<td>Mouse monoclonal IgG anti-PSA (mAb735)</td>
<td>1:1,000</td>
<td>Gift from Prof. Hildebrandt and Gerardy-Schahn in Hannover Medical School, Germany</td>
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<td>Mouse monoclonal IgM anti-PSA (5A5)</td>
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<td>Rabbit polyclonal anti-CD68 antibody</td>
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<tr>
<td>Mouse monoclonal IgG anti-neutrophils</td>
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<td><strong>Secondary antibodies</strong></td>
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<td>1:600</td>
<td>Jackson Immunoresearch</td>
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2.5.3 Terminal transferase dUTP nick-end-labeling (TUNEL) assay

For TUNEL assay, slides were washed 3 times for 15 min each in PBS in 0.2% Triton X-100 in PBS then treated with 20 μg/ml Proteinase K in 10 mM Tris, PH 7.5 and 5 mM EDTA at RT for 15 min to strip off nuclear proteins. After washing twice with PBS for 2 min each, slides were treated with equilibration buffer for 15 min. Then slides were then incubated with a reaction buffer containing terminal deoxynucleotidyl transferase (TdT, 0.3 unit/μl, Promega) for 60 min at 37 ºC. Slides were put in a coplin jar filled with wash/stop buffer (wash/stop buffer was diluted with distilled water at a ratio 1:34, Promega), agitated for 15 s and then incubated at RT for 10 min. After triple wash with PBS for 5 min each, slides were incubated with anti-digoxigenin conjugate solution (blocking solution:anti-digoxigenin rhodamine conjugate = 68:62) for 30 min at RT. DAPI was applied for 5 min incubation followed by thorough wash. The sections were coverslipped with 1:9 PBS-glycerol.

**Positive controls:** After Proteinase K treatment and quick wash, slides were incubated with DN buffer (30 mM Trizma base, PH 7.2, 4 mM MgCl₂, 0.1 mM DTT) at RT for 5 min. Slides were treated with DNase I (1000 U/ml) for 30 min at 15-25ºC to induce DNA strand breaks, and then followed by labelling procedure above.

**Negative control:** After Proteinase K treatment and quick wash, slides were incubated with reaction buffer without TdT, and then followed by labelling procedure above.

2.5.4 Flow cytometry Annexin V staining

Trypsinized SCs were centrifuged at 960 rpm for 8 min, the cells were washed in PBS once. SCs were then resuspended in 400 μl of Becton Dickinson (BD) Annexin V Binding Buffer (Cat. No.556454). Cells suspension were incubated with 2 μl Annexin V-FITC (BD, Cat. No. 556420), at RT for 15 min, then 5 μg/ml (final concentration) viability dye propidium
iodide was added. The samples were then subjected to flow cytometry.

2.6 Imaging

Sections were viewed under a Leica fluorescence microscope. Photographs were taken using a Hamamatsu (C-4742-95) digital camera (Herrsching, Germany) and Hipic software. Tetramethyl rhodamine isothiocyanate (TRITC), fluorescein isothiocyanate (FITC), amino methyl coumarin acetic acid (AMCA) and Alexa 647 or Cy-5 stained sections were viewed under the Leica Y3, L5, A4 and Y5 filter blocks, respectively. Some sections were viewed in a Zeiss confocal microscope (LSM 510).

2.7 Quantification and statistical analysis

**Quantification of SC culture purity:** Purity of all primary SC cultures was evaluated by the ratio of p75<sup>NTR</sup><sup>+</sup> or S100<sup>+</sup> cell number/DAPI<sup>+</sup> nuclei. Twenty fields were randomly captured under a 20× objective lens. Highly purified cultures (> 95%) and up to three passages were used in all SC experiments.

**Quantification of transduction efficiency of LV to SCs:** the transduction efficiency of LV/GFP was assessed by the ratio of GFP<sup>+</sup> cell number/DAPI<sup>+</sup> nuclei. Twenty fields were randomly captured under a 20× objective lens. The efficiency of transduction was 95 ± 2%. The co-transduction efficiency of LV/GFP and LV/PST-GFP was assessed by the ratio of GFP<sup>+</sup> cell number/PSA-ir<sup>+</sup> cell number. Twenty fields were randomly captured under a 20× objective lens. The efficiency of co-transduction was 90 ± 2%.

**Quantification of PSA-immunoreactivity on SCs in vivo:** images of GFP and PSA double-labelled transplanted cells at 7 or 30 days in 9 fields on 9 sections (3 sections from each of the 3 animals) were randomly captured on the basis of GFP fluorescence using a 63× oil immersion objective of a confocal microscope (Zeiss 510). All the parameters were kept constant for each session of measurements. Expression levels of PSA were estimated as the ratio of the area of PSA-ir over the area of GFP<sup>+</sup> cells using ImageJ software.
**Quantification of SCs survival in vivo:** to estimate the survival of transplanted SCs, the areas occupied by transplanted SCs (defined by GFP fluorescence) were measured in consecutive parasagittal sections of spinal cord (45 µm apart) with ImageJ software.

**Quantification of SCs migration in vivo:** the distances of 20 cells that spread the furthest from the epicenter of the transplants on three selected sections from each animal were measured with ImageJ software.

**Quantification of proliferation of SC or astrocytes in vitro:** to analyze the proliferation of GFP/SCs or PST/SCs in culture medium, five randomly selected fields per coverslip under a 20× objective lens of a Leica microscope were captured for counting. Six coverslips were used per experiment and the experiment was repeated 3 times. GFP/SCs or PST/SCs were identified with GFP fluorescence. The data are expressed as the ratio of double GFP+/BrdU+ cells over double GFP+/DAPI+ cells for the proliferation rate of SCs.

To analyze the proliferation of astrocytes in culture medium or in co-culture with GFP/SCs or PST/SCs, five randomly selected fields per coverslip under a 20× objective lens of a Leica microscope were captured for counting. Six coverslips were used per experiment and the experiment was repeated 3 times. Astrocytes were identified with a GFAP antibody. The data are expressed as the ratio of double GFAP+/BrdU+ cells over double GFAP+/DAPI+ cells for the proliferation rate of astrocytes.

**Quantification of expression level of p75NTR on SCs in vitro:** GFP/SCs or PST/SCs were plated on the coverslips and double immunostained for p75NTR (rabbit polyclonal IgG anti-p75NTR, 1:15,000) and PSA (mouse monoclonal IgG anti-PSA, mAb735, 1:2,500). Donkey anti-rabbit IgG TRITC (1:800) or goat anti-mouse IgG Alexa 647 (1:800) was used as the secondary antibodies. SCs were identified with GFP fluorescence. Mean grey value of the p75NTR-ir was measured under 40× objective lens of a Leica microscope on 120 randomly selected cells from each group using ImageJ software. The experiments were repeated twice.

**Quantification of infiltration of neutrophils or macrophage to SC grafts:** to assess the
infiltration of neutrophils or macrophages, six randomly selected fields from two sections of each animal (n = 3) under a 10× objective lens of a Leica microscope were captured for measurement. The ratio of the area occupied by infiltrated neutrophils or macrophages within the SC grafts over the area occupied by the transplanted SCs was calculated as an index for the level of inflammatory cell.

All the data were represented as mean ± S.E. Statistical significance was determined using two-tailed Student’s t-test.
Chapter 3
Engineered expression of PSA improves Schwann cell survival after transplantation into the spinal cord

3.1 Abstract
Poor survival is a common phenomenon for transplanted cells. It has been reported the majority of cells dies within the first week due to necrosis and apoptosis. In the current study SCs were transplanted both in uninjured or injured spinal cord with or without immunosuppression. It was found that without ciclosporin treatment, more PST/SCs survived at 7, 19 and 30 days after transplantation compared with GFP/SCs. Ciclosporin treatment significantly increased the survival of GFP/SCs but has no effect on the survival of transplanted PST/SCs. Similarly, in a spinal cord crush injury model, when GFP/SCs or PST/SCs were transplanted caudal to the lesion site with ciclosporin treatment daily, no marked difference was found in the overall survival of SCs between these two groups 3 weeks after transplantation. These data indicate PSA expression on SCs may have similar effect to ciclosporin treatment in protecting grafted SCs from host immune response. Moreover, with ciclosporin treatment, still more PST/SCs survived than GFP/SCs at early stage (7 days after transplantation). Proliferation and apoptosis of transplanted SCs were examined to see whether these two factors were accounted for the enhanced survival of PST/SCs. BrdU incorporation assay showed no proliferation of grafted SCs in both groups 19 days after transplantation, indicating that the difference in the survival between PST/SCs and GFP/SCs was not due to increased proliferation of PST/SCs. Also no difference was found in TUNEL$^+$ SCs between grafted GFP/SCs and PST/SCs 1 day, 7 and 19 days after transplantation. However, we found much lower p75$^{NTR}$-ir in PST/SCs compared with that in GFP/SCs in vitro by immunocytochemistry. It has been reported that p75$^{NTR}$ is involved in SC death both in vitro and in vivo. We speculate PSA induced down-regulation of p75$^{NTR}$ expression on SCs may partially contribute to the enhanced survival of PST/SCs.
3.2 Aims
In this study, we investigated the effect of PSA expression on (1) the survival of SCs both in uninjured and injured spinal cord \textit{in vivo}; (2) SC proliferation both \textit{in vivo} and \textit{in vitro}; (3) apoptosis of SCs after grafting; (4) recruitment of inflammatory cells to the SC grafts at early stage and (5) expression level of p75$^\text{NTR}$ in SCs.

3.3 Results
3.3.1 Purity of cultured Schwann cells and efficiency of lentiviral vector transduction of Schwann cells \textit{in vitro}.
SCs were isolated from the sciatic nerves and brachial plexuses of neonatal rats. In culture, the majority of the dissociated cells displayed bipolar or tripolar shape, which is typical for cultured SCs. A very small proportion of cells displayed fat or polygonal shape, which was recognized as fibroblasts. The purity of SCs was assessed by using p75$^\text{NTR}$ or S100 immunostaining. The ratio of p75$^\text{NTR}^+$ or S100$^+$ cells over DAPI stained nuclei was calculated from photomicrographs using a 20× objective lens, which remained at 98 ± 0.3% throughout the period of the study (SCs of up to three passages were used in all the experiments) (Fig. 3-1A-C). Transduction of SCs with LV/GFP resulted in robust GFP expression. Transduction efficiency was calculated as the ratio of GFP$^+$ cells over DAPI stained nuclei, which stood at 95 ± 2% at MOI (multiplicity of infection) of 15 (Fig. 3-2A-C).
Fig. 3-1 Purity of cultured Schwann cells.
(A) Cultured SCs double stained with anti-p75\textsuperscript{NTR} (A) and anti-S100 antibody (B). (C) Nuclei of cultured cells stained with DAPI. (D) Merged image of (A) (B) and (C). Scale bar = 50 µm.

Fig. 3-2 Efficiency of lentiviral vector mediated transfer of GFP to Schwann cells.
(A) SCs expressed high level of GFP protein 5 days after being transduced with LV/GFP. (B) Nuclei of cultured cells were stained with DAPI. (C) Merged image of (A) and (B). Scale bar = 100 µm.
3.3.2. Expression of PSA on LV/PST-GFP transduced Schwann cells in vitro.

PSA immunoreactivity (PSA-ir) was hardly detectable in LV/GFP transduced SCs. Very low level of PSA-ir was found on a few LV/GFP transduced SCs (Fig. 3-3C1-C3). In contrast, LV/PST-GFP transduced SCs displayed strong PSA-ir on the cell surface of unpermeabilized cells (Fig. 3-3D1-D3). Although the PSA-ir was present all over the cell bodies and the processes, granules with high-density of PSA-ir were a common feature. Such PSA-ir profile is similar to those reported before (Arellano et al., 2002). The expression of PST-GFP fusion protein was demonstrated by the GFP fluorescence on the Golgi apparatus (Fig. 3-3A) where they exert their biological functions. The expression of PSA in LV/PST-GFP transduced SCs was also confirmed by immunoblotting (Fig. 3-3B). A dense smear with molecular mass over 150 KD was identified in the lane of LV/PST-GFP transduced SCs. The molecular mass corresponds to that of polysialylated NCAM. The band was absent after the treatment with Endo-N, indicating the specificity of the antibody for PSA. No PSA specific band was detected in naïve SCs. For easy identification of transplanted SCs, SCs were co-transduced with LV/PST-GFP and LV/GFP. The co-transduction efficiency was over 90%.
Fig. 3-3 Detection of PSA expression on PST/SCs in vitro.

(A) The expression of PST-GFP fusion protein was demonstrated by the GFP fluorescence. Arrows point to the location of PST-GFP fusion protein. (B) Immunoblotting image shows a band of polysialylated NCAM in the lane of LV/PST-GFP transduced SCs and the band was absent after the treatment with Endo-N. No PSA specific band was detected in naïve SCs. (C1-3) Low level of PSA-ir present in a few SCs transduced by LV/GFP. (D1-3) Extensive and high-level expression of PSA was detected on SCs co-transduced by LV/PST-GFP and LV/GFP. Scale bar for A = 10 µm; scale bar for C and D = 50 µm.
3.3.3 Down-regulation of PSA expression on LV/PST-GFP transduced Schwann cells in vivo.

In order to define the time window of PSA expression on the grafted PST/SCs, we analyzed animals receiving PST/SCs at 7 and 30 days after transplantation. Images of GFP and PSA double-labeled transplanted cells at 7 or 30 days in 9 fields of view on 9 sections (3 sections from each of the 3 animals) were randomly captured using a 63× oil immersion objective of a confocal microscope (Zeiss 510). All the parameters were kept constant for each session of measurements. Expression levels of PSA were estimated as the percentage of the area of PSA-ir over the area of GFP+ cells using the image analysing software ImageJ. Most of the grafted PST/SCs expressed PSA at 7 days after transplantation (77.6 ± 6.9%, PSA-ir vs. GFP fluorescence) (Fig. 3-4A-C). The expression dropped to a lower level at 30 days (44.8 ± 3.7%) (Fig. 3-4D-F).

Fig. 3-4 Detection of PSA expression on Schwann cell grafts in vivo.
PSA-ir was remained detectable on PST/SCs at 7 days (A-C) and 30 days (D-F) post transplantation by using the antibody mAb735 antibody. Arrows pointed to the PSA-expressing SCs. Scale bar = 10 µm.
3.3.4 Survival of grafted Schwann cells after transplantation into the uninjured spinal cord.

3.3.4.1 Survival of Schwann cells after transplantation into uninjured spinal cord without ciclosporin treatment.

GFP/SCs or PST/SCs were transplanted into T8 level of rat spinal cord. One day after SCs transplantation many bright green cells with oblong and round profiles were found in both GFP/SCs and PST/SCs grafts (Fig. 3-5A-B). Cells with such profiles were dead cells as cells shrank significantly so that GFP florescence condensed inside the cells to make them appear very bright. Dead cells were hardly visible in 7-days grafts. The dead green cells made quantification of cell survival impossible in 1-day grafts by using ImageJ to measure the areas occupied by GFP/SCs. Therefore, only animals from 7 days upwards were used for comparing the survival of transplanted SCs between PST/SCs and GFP/SCs groups.

Fig. 3-5 Transplanted Schwann cells in host spinal cord 1 day after transplantation. Images show that many bright green dead cells with oblong and round profiles were found in the injection site in both GFP/SCs (A) and PST/SCs grafts (B) one day after SCs transplantation. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm.
The total areas occupied by transplanted SCs (identified with GFP fluorescence) were measured with ImageJ to assess the survival of transplanted SCs. The level of survival for GFP/SCs and PST/SCs were 0.50 mm$^2$ vs. 1.88 mm$^2$; 0.33 mm$^2$ vs. 1.00 mm$^2$ and 0.08 mm$^2$ vs. 0.31 mm$^2$, $p < 0.01$ or $p < 0.05$, Fig. 3-8A-C) at 7, 19, and 30 days respectively after transplantation. Significantly more PST/SCs survived than GFP/SCs over these 3 time points after transplantation (compare Fig. 3-6A-C).
Fig. 3-6 Survival of Schwann cells after transplantation into uninjured spinal cord without ciclosporin treatment.
Consecutive parasagittal sections of spinal cord illustrating the transplanted GFP/SCs (A) and PST/SCs (B) 7 days after transplantation. (C) Areas occupied by transplanted SCs in serial parasagittal sections of spinal cord measured using ImageJ. *p < 0.05; **p < 0.01. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 8.
3.3.4.2 Survival of Schwann cells after transplantation in uninjured spinal cord with ciclosporin treatment.

In order to exclude the effect of immune rejection of the transplanted SCs, some animals received ciclosporin injection daily. Similar to the untreated groups, bright green dead cells were found in the injection site in both GFP/SCs and PST/SCs grafts 1 day after transplantation (Fig. 3-7A-B).

Fig. 3-7 Transplanted Schwann cells in host spinal cord with ciclosporin treatment 1 day after transplantation.
Images show that bright green dead cells with oblong and round profile were found in both GFP/SCs (A) and PST/SCs grafts (B) 1 day after SCs transplantation. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm.

Significantly more PST/SCs survived 7 days after transplantation comparing with GFP/SCs (0.88 ± 0.17 mm² vs. 2.09 ± 0.54 mm², p < 0.05, Fig. 3-8A-C). However, there is no difference between the two groups 30 days after transplantation (0.38 ± 0.05 mm² vs. 0.40 ± 0.06 mm², p > 0.05, Fig. 3-8C). Ciclosporin treatment increased the survival of transplanted GFP/SCs both 7 days and 30 days after transplantation, but it did not have significant effect on the overall survival of transplanted PST/SCs (Fig. 3-8D).
Fig. 3-8 Survival of Schwann cells with ciclosporin treatment.
Consecutive parasagittal sections of spinal cord illustrate the transplanted GFP/SCs (A) and PST/SCs (B) 7 days after transplantation. (C) Graph shows the areas occupied by transplanted SCs measured in serial sections of spinal cord using ImageJ. (D) Graph shows the survival level of transplanted SCs with/without ciclosporin treatment. *$p < 0.05$; **$p < 0.01$. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 8.
3.3.4.3 Survival of Schwann cells after transplantation in injured spinal cord with ciclosporin treatment.

In order to investigate whether PSA expression could enhance the survival of SCs grafts in injured spinal cord, GFP/SCs or PST/SCs were transplanted 2.5 mm caudal to a hemi-curshed injury site of spinal cord. Animals were treated with ciclosporin daily and sacrificed 3 weeks post transplantation. By measuring the total area occupied by transplanted SCs in serial parasagittal sections of spinal cord, no difference was found between these two groups (0.49 mm$^2$ vs. 0.71 mm$^2$ for GFP/SCs and PST/SCs group respectively. $p > 0.05$, Fig. 3-9)

![Survival of Schwann cells after transplantation in injured spinal cord.](image)

Fig. 3-9 Survival of Schwann cells after transplantation in injured spinal cord. Total areas occupied by transplanted SCs were measured in serial sections of spinal cord using ImageJ. There is no difference in overall survival of the SCs between these two groups. n = 8.

3.3.5 Assessment of the proliferation of transplanted Schwann cells in the spinal cord using BrdU incorporation assay.

After we have found that more PST/SCs survived than GFP/SCs after transplantation, we wanted to know whether it was partially due to a higher proliferation degree of PST/SCs. We first tested whether PSA expression on SCs had any effect on the proliferation of SCs in vitro. High proportions of SCs in both groups were BrdU$^+$, indicating the cells were proliferating vigorously. However, no difference was detected in the proportions of BrdU$^+$ nuclei between GFP/SCs and PST/SCs (54.3% vs. 53.7%, $p > 0.05$, Fig. 3-10).
Fig. 3-10 Assessment of proliferation of PST/SCs and GFP/SCs in vitro using BrdU incorporation assay.
SCs transduced with LV/GFP (A) or LV/PST-GFP (B) immunostained with anti-BrdU antibody. (C) Graph shows that there is no difference in BrdU incorporated nuclei between the two groups. Scale bar = 25 µm. n = 3.

To check the proliferation of the transplanted SCs, four animals from each group receiving GFP/SCs or PST/SCs transplantation were given BrdU injection for 3 days just before they were sacrificed. Hardly any GFP/SCs (Fig. 3-11A-D) or PST/SCs (Fig. 3-11E-H) were found to incorporate BrdU 19 days after grafting. Some BrdU+ cells were observed close to the transplanted SCs (Fig. 3-11B, 11F) which were probably reactive astrocytes as they were GFP negative.
Fig. 3-11 BrdU incorporation for transplanted Schwann cells in spinal cord.
GFP/SCs (A-D) or PST/SCs (E-H) immunostained with anti-BrdU antibody (red in B, F) and DAPI (C, G) 19 days after being grafted into rat spinal cord. (D) and (H) are merged images of (A, B, C) and (E, F, G) respectively. Arrowheads point to the BrdU incorporated cells. These cells appeared GFP negative. Scale bar = 100 µm.
3.3.6 d-UTP-digoxigenin nick end-labeling (TUNEL) assay.

TUNEL staining was used to identify apoptosis of transplanted SCs. At 1 day (Fig. 3-12C-D; 12G-H) and 7 days (Fig. 3-13C-D; 3-13G-H) after SCs transplantation a few PST/SCs and GFP/SCs were found to be TUNEL+. The numbers of TUNEL+ cells were similar between PST/SCs and GFP/SCs. At 19 days post transplantation TUNEL+ cells were hardly detectable (Fig. 3-14C-H; 14G-H).
Fig. 3-12 TUNEL assay for Schwann cells grafts 1 day post transplantation. GFP/SCs (A-D) and PST/SCs (E-H) 1 day after being grafted into rat spinal cord. Nuclei were stained with DAPI (B, F), TUNEL\(^+\) nuclei are shown in red (C, G). (D) and (H) are merged images of (A, B, C) and (E, F, G) respectively. Arrows point to the TUNEL\(^+\) SCs. Scale bar = 100 µm. n = 2.
Fig. 3-13 TUNEL assay for Schwann cell grafts 7 days post transplantation. GFP/SCs (A-D) and PST/SCs (E-H) 7 days after being grafted into rat spinal cord. Red for TUNEL⁺ nuclei. (C, G). Nuclei were stained with DAPI (B, F). (D) and (H) are merged images of (A, B, C) and (E, F, G) respectively. Scale bar = 100 µm. n = 2.
Fig.3-14 TUNEL assay for Schwann cells grafts 19 days post transplantation. GFP/SCs (A-D) and PST/SCs (E-H) 19 days after being grafted into rat spinal cord. Red is for TUNEL staining (C, G). Nuclei were stained with DAPI (B, F). (D) and (H) are merged images of (A, B, C) and (E, G, F) respectively demonstrating the apoptosis of transplanted GFP/SCs in spinal cord; TUNEL^+ cells are hardly detectable on GFP/SCs or PST/SCs at 19 days after transplantation. Scale bar = 100 µm.
3.3.7 Recruitment of inflammatory cells at Schwann cell transplantation sites in uninjured spinal cord in early stages.

As shown previously, the majority of cell death after transplantation occurs in the first week. During the process of transplantation, the blood-spinal cord barrier is disrupted which leads to an inflammatory response that enhances cell death. Neutrophil and macrophage are the main type of inflammatory cells that infiltrate the spinal cord at the early stage after transplantation. Three animals receiving GFP/SCs or PST/SCs transplantation without ciclosporin treatment were used in this study. To assess the infiltration of neutrophils or macrophages, the proportion of the area occupied by infiltrated neutrophils or macrophages within the transplanted SCs grafts over the area occupied by the transplanted SCs was calculated to assess the level of inflammatory cell infiltration. It was found that the presence of neutrophil infiltration around SC transplants was obvious at 1 day and 7 days post transplantation (Fig. 3-15, 3-16). However, there is no difference between PST/SCs and GFP/SCs groups in the level of neutrophil infiltration (Fig. 3-15, 3-16).
Fig. 3-15 Assessment of neutrophil infiltration 1 day post transplantation in uninjured spinal cord.

GFP/SCs (A) and PST/SCs (D) grafts immunostained with a specific antibody for neutrophils (B, E) 1 day post transplantation. (C) and (F) are merged images of (A) and (B), (D) and (E) respectively. (G) Proportion of the areas occupied by neutrophils over the area occupied by transplanted SCs as an index for the level of neutrophil infiltration. No difference was found between GFP/SCs and PST/SCs groups. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 3.
Fig. 3-16 Assessment of neutrophils infiltration 7 days post transplantation in uninjured spinal cord.

Neutrophil infiltration at the transplantation site 7 days post transplantation of GFP/SCs (A) or PST/SCs (D) in intact spinal cord. SC grafts were immnostained with a neutrophil antibody (B, E). (C) and (F) are merged images of (A) and (B), (D) and (E) respectively. (G) Proportion of the areas of occupied by neurophils over the area occupied by transplanted SCs as an index for the level of neutropil infiltration No difference was found between GFP/SCs and PST/SCs groups. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 3.
Like neutrophils, many ED1\(^+\) macrophages were also present in or around the transplanted cells 1 day after transplantation (Fig. 3-17). However, no difference was found in the levels of ED1\(^+\) cells at the transplantation sites between PST/SCs and GFP/SCs groups 1 day after transplantation. In contrast to neutrophils, only a few macrophages/microglias were present at the transplantation site 7 days after transplantation (Fig. 3-18). No quantification was performed to compare the levels of ED1\(^+\) cells at the transplantation sites between the PST/SCs and GFP/SCs groups at this time point due to negligible level of ED1\(^+\) cells.
Fig. 3-17 Assessment of macrophage infiltration 1 day post transplantation in uninjured spinal cord.
GFP/SCs (A) and PST/SCs (D) grafts immnostained with a specific antibody for macrophage ED1 (B, E) 1 day post transplantation. (C) and (F) are merged images of (A) and (B), (D) and (E) respectively. (G) Proportion of the areas of occupied by ED1+ cells over the area occupied by transplanted SCs as an index for the level of macrophage infiltration. No difference was found between GFP/SCs and PST/SCs groups 1 day post transplantation. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 3.
Fig. 3-18 Assessment of macrophage infiltration 7 days post transplantation in uninjured spinal cord.

GFP/SCs (A) and PST/SCs (D) grafts immunostained with a specific antibody for macrophage, ED1 (B, E) 7 day post transplantation. Only few macrophages were detected in both groups. (C) and (F) are merged images of (A) and (B), (D) and (E) respectively. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 3.
### 3.3.8 PSA expression regulates p75<sup>NTR</sup> expression on cultured Schwann cells.

It has been reported that immature neurons lacking PSA-NCAM expressed significantly higher level of p75<sup>NTR</sup> than the cells with PSA-NCAM. Removal of PSA from NCAM induced an enhanced activation of p75<sup>NTR</sup> signalling pathways and led to increased cell death. It was suggested that by limiting p75<sup>NTR</sup> expression, PSA-NCAM might protect newborn neurons in the developing stage (Gascon et al., 2007). Thus, it is possible that PSA expression in PST/SCs may induce the down-regulation of p75<sup>NTR</sup> expression on transplanted SCs and enhance their survival. In this study, we tested the expression level of p75<sup>NTR</sup> on both PST/SCs and GFP/SCs by immunocytochemistry using a series of diluted anti-p75<sup>NTR</sup> antibody. With the dilution of 1:12,000 or 1:15,000 the difference in the levels of p75<sup>NTR</sup>-ir between PST/SCs and GFP/SCs became obvious (compare Fig. 3-19B against F). Mean grey value of p75<sup>NTR</sup>–ir was measured on 120 randomly selected cells using ImageJ to assess the p75<sup>NTR</sup> expression level. It was found that there was much lower level of p75<sup>NTR</sup>–ir present in PST/SCs compared with GFP/SCs (p < 0.001) (Fig. 3-19I). 


Fig. 3-19 Assessment of the expression level of p75<sup>NTR</sup> on Schwann cell in vitro.
Expression of p75<sup>NTR</sup> in GFP/SCs (A-D) and PST/SCs (E-H) in vitro. Weak p75<sup>NTR</sup>–ir (red) was presented in LV/PST-GFP transduced PSA<sup>-</sup> (blue in G, H) SCs compared with LV/GFP transduced PSA<sup>+</sup> SCs (C, D). SCs were identified with GFP fluorescence. (I) Quantification of mean grey value of p75<sup>NTR</sup>-ir of PST/SCs and GFP/SCs. Significantly much lower level of p75<sup>NTR</sup>–ir was found in PST/SCs compared with that in GFP/SCs. ***p < 0.001. Scale bars = 25 µm. Experiments were repeated twice.
3.4 Discussion

Although SCs transplanted into the CNS can promote axonal regeneration and remyelination and improve functional recovery in animal models of spinal cord injury, poor survival of transplanted SCs in the CNS is a common problem and limits their therapeutic potential. It has been reported that 78% reduction in SC number within the first week after transplantation was mainly due to necrosis, and only around 15% of SCs survived 28 days after transplantation (Hill et al., 2007). Such trend of SC death was confirmed in our study and we also observed that TUNEL\(^+\) cells were surprisingly low at 1 day and 7 days post transplantation given the extent of cell death, indicating that necrosis was the main type of cell death. The second wave of SC death occurred after they have integrated into the spinal cord tissue could result from transplant rejection since the survival of transplanted SCs can be enhanced by immunosuppression (Hill et al., 2006). In the current study we also showed that ciclosporin treatment improved the survival of GFP/SCs by 4.6-fold at 30 days, indicating immune rejection plays a major role in cell loss at the late stage. However, without immunosuppression, we found PST/SCs survived better than GFP/SCs at all three time points, 7, 19 and 30 days after transplantation. With ciclosporin treatment, the significant difference between the PST group and GFP group in survival only existed at the early stage (7 days); no difference was seen between the two groups at 30 days after transplantation. The result indicates that ciclosporin can significantly improve the survival of GFP/SCs over the time, but has little effect on the survival of PST/SCs. The effects of PSA on the survival of transplanted SCs without ciclosporin treatment is similar to the effect of ciclosporin on transplanted GFP/SCs, especially at the late stage, indicating PSA may have certain immunoprotective effects for transplanted cells.

We have examined several potential factors that might have contributed to the better survival of PST/SCs. First, we investigated whether PST/SCs have a higher level of proliferation. Cultured SCs in both groups showed high population of BrdU\(^+\) cells, however, there was no difference between these two groups. BrdU staining showed there was no obvious cell proliferation in both groups at 19 days post transplantation. Thus, the improved survival of PST/SCs was not due to cell expansion after grafting.

TUNEL staining on 1, 7, 19 days after transplantation showed that there were more
TUNEL+ cells on 1 day, whereas, much less on 19 days, although the number of stained cells in tissue sections was quite small even 1 day after transplantation. No difference was found in the numbers of TUNEL+ cells in these two groups. However, we cannot draw the conclusion that PSA over-expression does not rescue SC from apoptosis based on the negative TUNEL staining results due to the narrow time window for the apoptosis detection using TUNEL assay.

In this study, recruitment of macrophage and infiltration of neutrophils to the transplantation site were assessed with immunohistochemistry by using another set of spinal sections from animals without ciclosporin injection. However, there is no difference between PST/SCs and GFP/SCs groups in the levels of neutrophil or macrophage infiltration 1 and 7 days after transplantation which indicates PSA over-expression on SCs does not reduce the recruitment of inflammatory cells at SC transplantation sites at early stage. It has been reported that sialic acids have anti-recognition effect. They can shield antigenic sites and thus weaken the immuno-reactivity. Sialic acids render cells as “self”, not allowing recognition by the immune system or by, e.g., macrophage lectins (Schauer, 2004). The antirecognition effects of sialic acids are explained by their negative charges in combination with a bulky, hydrophilic molecule (Kelm and Schauer, 1997). After sialidase treatment of mammalian erythrocytes and re-injecting them back to the bloodstream, most of the modified cells disappeared from the bloodstream within a few hours, although their normal survival time in humans is about 120 days (Janicik et al., 1978). These cells were phagocytosed by liver Kupffer cells, spleen, or peritoneal macrophages. It is reasonable to propose that expression of PSA on SCs may shield the antigenic sites on the cells and thus diminish the immune response from the host. The masking effect of PSA has been explored to protect therapeutic peptides, enzymes, and other proteins from being removed from circulation after administration so as to prolong their effects and reduce the dosage (Gregoriadis et al., 2000).

In addition, we also found that the survival of PST/SCs in animals without ciclosporin treatment was still significantly better than GFP/SCs in animals with ciclosporin treatment at 7 days, indicating PSA may have other potential mechanisms to protect transplanted SCs from death apart from the purported immunoprotective effects.
It has been reported that immature neurons lacking PSA-NCAM expressed significantly higher level of \( p75^{\text{NTR}} \) than the cells with PSA-NCAM. Removal of PSA from NCAM induced an enhanced activation of \( p75^{\text{NTR}} \) signalling pathways and led to increased cell death. It was suggested that by limiting \( p75^{\text{NTR}} \) expression, PSA-NCAM might protect newborn neurons in the developing stage (Gascon et al., 2007). It has also been demonstrated that TNF\( \alpha \), one of the potent cytotoxic molecules activated during inflammation, can mediate SC death by up-regulating \( p75^{\text{NTR}} \) expression (Boyle et al., 2005). Indeed, we did find the expressing level of \( p75^{\text{NTR}} \) in PST/SCs was significant lower than that in GFP/SCs. Thus, we proposed PSA expression in PST/SCs may down-regulate expression of \( p75^{\text{NTR}} \) on transplanted SCs and enhance their survival, which could be one of the mechanisms to improve the survival of PST/SCs compared with GFP/SCs.

There are several predominant pathways that lead to SC death during/after transplantation. The first stress the cells encounter during the transplantation process is the lack of matrix support, which begins before transplantation. Typical cell preparations for injection involve enzymatically dispersed cells suspended in protein-free medium, and stored on ice. During this period, important adhesion-related survival signals such as laminin could be absent and not reinitiated for many hours until the cells find themselves in the context of a recipient spinal cord and even then, the proper basal surface for the cells may not be present (Koda et al., 2008). Serum and trophic factor deprivation is another issue. LPA in serum has been proved to support SC survival \textit{in vitro} (Li et al., 2003c) and growth cocktail used in SC culture includes several types of factors which have been shown to boost SCs survival and proliferation (Dong et al., 1995; Gavrilovic et al., 1995; Syroid et al., 1996). Large amount of released ATP or glutamate from the damaged cells also may activate their own receptors to induce toxicity on transplanted SCs. These factors will be discussed in \textbf{Chapter 4}.

In conclusion, PSA over-expression enhances the survival of transplanted SCs to a certain degree which may be related to protecting cells from the attack of host immune system and the down-regulation of \( p75^{\text{NTR}} \).
Chapter 4

Investigation of the molecular mechanisms underlying Schwann cells death after transplantation

4.1 Abstract
Poor cell survival is one of factors potentially limiting the efficacy of the cell transplantation therapy. The current study tested several potential factors contributed to SC death after grafting. It was found that high concentration of glutamate (1 mM) did not induce SC death. Short-term (up to 5 h) serum and growth factor withdrawal did not cause obvious morphological change of SCs. Prolonged serum and growth factor withdrawal (over 26 h) caused significant SC death. Interestingly, PSA expression can partially protect SCs from death induced by serum and growth factor withdrawal. In the current study we confirmed that SCs from rats or mice expressing P2X7Rs using immunocytochemistry and immunohistochemistry. High-concentration of ATP has been reported to induce death of certain types of cells by activating P2X7R. ATP was found to induce SC death on a concentration-dependant manner. Cell death caused by ATP can be blocked by an irreversible P2X7R antagonist, oxidized ATP (oxATP), confirming that ATP induced SCs death is mediated by P2X7Rs. Moreover, PSA expression can also partially protect SCs from ATP induced cell death in vitro. In addition, SCs pretreated with oxATP before transplantation showed 38.2% more SCs survived than that of untreated SCs one week after transplantation into the intact spinal cord. Taken together, these results indicate that targeting P2X7R on SCs could be a potential therapeutic strategy to prevent cell death after transplantation.

4.2 Aims
The process of cell transplantation is accompanied with injury to the transplantation site and vessel breakage, which causes large amount of glutamate and ATP release to the extracellular space at the injection site. Glutamate receptors have been reported to be expressed on SCs (Dememes et al., 1995; Fink et al., 1999), but it is not known whether glutamate can induce SC. Serum and several growth factors used in the SC culture
medium have shown to enhance SC survival (Dong et al., 1995; Gavrilovic et al., 1995; Syroid et al., 1996). It has been speculated serum and growth factors withdrawal plays an important role in the early necrosis and apoptosis of transplanted SCs (Hill et al., 2007). P2X7R activation by high concentration of extracellular ATP can induce the death of several types of cells (Hogquist et al., 1991; Perregaux and Gabel, 1994; Falzoni et al., 1995; Ferrari et al., 1999; Tsukimoto et al., 2005). P2X7R expression on mouse SCs has been demonstrated by electrophysiological and immunohistochemical studies (Colomar and Amedee, 2001). In this study, we tested the effects of (1) glutamate; (2) serum and growth factor withdrawal; (3) ATP on the survival of SC in vitro, and (4) whether blockade P2X7Rs using oxATP can rescue the SC death induced by ATP both in vitro and in vivo.

4.3 Results

4.3.1. Glutamate does not induce significant Schwann cell death in vitro.

We first tested the effect of glutamate on the survival of SCs. 0.1 mM or 1 mM (final concentration) glutamate was added into the culture dish for up to 1 h. By direct observation under a microscope, there was no obvious cell morphology change was found either when cell incubated with 0.1 or 1 mM glutamate for 1 h. Flow cytometry apoptosis assay results showed 94.1 ± 2.3%, 95.3 ± 2.8% and 94.6 ± 1.7% of live cells in culture medium (as control), 0.1 mM, and 1 mM glutamate treated group respectively. No significant difference in cell survival was found between these three groups, indicating SCs are insensitive to high concentration of glutamate (Fig. 4-1).
Fig. 4-1 Effect of glutamate on the survival of Schwann cells in vitro.
Phase contrast images show no obvious morphology change when SCs exposed to 0 (A), 0.1 mM (B) and 1 mM glutamate (C) for 30 min. (D) Flow cytometry apoptosis assay shows the similar portion of live cells in these three groups 1 h after glutamate treatment. (E) Graph shows percentage of live cells using flow cytometry apoptosis assay. Glu: glutamate. Experiments were repeated three times.
4.3.2. Serum and growth factor withdrawal induce Schwann cells death *in vitro.*

Long-term (> 26 h) serum withdrawal has been shown to induce SC and OEC death *in vitro* (Weiner and Chun, 1999; Zhao et al., 2007). For all SC transplantation experiments in this study, SCs were resuspended in DMEM medium without both serum and growth factors. To mimic the *in vivo* environment that transplanted SCs may encounter, we tested the effect of both serum and growth factors withdrawal on the survival of SCs *in vitro.* No significant cell death was observed under microscope 5 h after serum and growth factor withdrawal, indicating SCs can withstand serum- and growth factors-free condition for a few hours. After prolonged (26 h) serum and growth factors withdrawal the processes of SCs shrank and became slimmer, but it was difficult to tell the difference between the two groups under microscope (Fig.4-2C, F). However, flow cytometry apoptosis assay showed more PST/SCs were alive than GFP/SCs (51.1 ± 1.3% vs. 38.2 ± 1.8%, *p* < 0.01, Fig. 4-2). The data indicates PSA expression may protect SCs death from serum and growth factors withdrawal to a certain degree.
Fig. 4-2 Effect of serum and growth factor withdrawal on the survival of Schwann cells in vitro.
Phase contrast images show no obvious morphology change of SCs 5 h after serum and growth factor withdrawal (B, E) compared with SCs in normal culture medium (as control) (A, D). Long-term (26 h) serum and growth factor withdrawal resulted in the morphology change of SCs. They became shranked and slimmer in the both groups (C, F). (G) Graph shows the percentage of live cells 26 h after serum and growth factor withdrawal measured using flow cytometry cell apoptosis assay. Scale bar = 100 µm. **p < 0.01. Experiments were repeated three times.

4.3.3 ATP induces Schwann cell death in vitro.
P2X7R is known as the receptor subtype that mediates high-concentration ATP induced cell death. In the previous study, P2X7R has been shown to be localized on mouse SCs by electrophysiology and immunocytochemistry (Colomar and Amedee, 2001).
(1) Schwann cells express P2X7Rs

In this study, we first examined the expression of P2X7R on the rat SCs in culture as well as in sciatic nerves with immunohistochemistry. The most widely used antibody for P2X7R is a polyclonal anti-P2X7R from Alomone (Jerusalem, Israel, Cat. number: APR-004, with a dilution of 1:70). In order to further assure the specificity of the antibody, sciatic nerves from C57/Bl6 wild-type and P2X7R knockout mice (kindly provided by GSK) were stained with the same antibody. In vitro P2X7R immunoreactivity (P2X7R-ir) was shown to be distributed inside cytoplasm of S100+ SCs (Fig. 4-3A-C). P2X7R-ir was not detected in cultured astrocytes (Fig. 4-3D-F), which may account for their resistance to ATP induced death (see Fig.4-8).

![Image: Fig. 4-3 Detection of P2X7Rs on Schwann cells and astrocytes.](image)

(A) Expression of P2X7Rs on cultured SCs (A-C). SCs were identified with a SC marker S100 (green in A, C). In contrast to SCs, no P2X7R-ir (red) was detected on cultured astrocytes (D-F). Astrocytes were identified with an antibody against GFAP (green in D, F). Scale bar = 50 μm.

The specificity of the P2X7R antibody was confirmed since P2X7R-ir was not detected on the sciatic nerve from P2X7R knockout mice (Fig. 4-4B), while strong P2X7R-ir was detected on sciatic nerve from the wild-type mice (Fig. 4-4A). Co-localization of P2X7R and S100 immunosignals confirmed the presence of P2X7R on SCs (Fig. 4-4D-E).
**Fig. 4-4 Detection of P2X7Rs on mouse sciatic nerve.**
Expression of P2X7Rs on mouse sciatic nerve from wild-type mice (A) and P2X7R knockout mice (B) stained with an anti-P2X7R antibody. SCs were identified with a SC marker S100. High magnification images show P2X7R mainly located on the nodes of Ravier (Arrows in C-E). Scale bar = 50 µM in A-B and 10 µM in C-E. WT: wild-type, KO: knockout.

**ATP induced cultured Schwann cell death is dose-dependent**
We found that exposure of SCs to high concentrations (over 3 mM) of ATP led to significant cell death *in vitro* (Fig. 4-5). Morphological change of SCs occurred within 15 min after exposure to 5 mM ATP, demonstrated by withdrawal of cell processes and rounded-up of cells bodies. Most of SCs detached from the culture dish after 1 h exposure to 5 mM ATP. By using flow cytometry apoptosis assay kit to measure the cell death, it was shown that 73.9 ± 5.6%, 63.2 ± 4.5%, and 52.3 ± 2.9% cells were alive after being exposed to 3, 4, 5 mM ATP respectively. Significantly more cells were dead with ATP increasing concentrations (*p* < 0.05 or *p* < 0.001).
Fig. 4-5 ATP induces Schwann cell death in vitro.
Phase contrast images show SCs in culture before (A) and after exposure to 0.1 mM ATP (B) or 5 mM ATP (C) for 30 min. (D) Flow cytometry shows the proportions of live cells after exposure to 3, 4, 5 mM ATP for 1 h. (E) Graph illustrates the percentage of live cells with the increasing concentrations of ATP. *p < 0.05, ***p < 0.001, one-way ANOVA. Experiments were repeated three times.
To further confirm whether P2X7R activation can induce SC death, BzATP, the most potent agonist for P2X7R was added to the culture medium and the morphology change of cells was observed under microscope. It was found that cells in the dish containing 200 µM BzATP started to shrink and round up within 15 min. By 30 min, nearly all the cells in the BzATP dish became rounded. After incubation for 1 h, cells were dissociated and subjected to flow cytometry apoptosis assay. 68.7 ± 2.3% cells were alive after exposure to BzATP, in comparison with 95.1 ± 2.7% live cells in the control dish (Fig. 4-6, p < 0.01), indicating that P2X7R activation cause SC death.

Fig. 4-6 Schwann cell death induced by BzATP.
Flow cytometry apoptosis assay shows that the percentage of live cells in culture medium (A) or after exposure to 200 µM BzATP (B) for 1 h. (C) Graph illustrates percentage of live cells after BzATP treatment. **p < 0.01. Experiments were repeated three times.

(3) ATP does not induce fibroblasts or astrocytes death in vitro
SC cultures were normally contaminated with a few fibroblasts. When exposed to 5 mM ATP, the majority of SCs rounded up or detached from the culture dishes while the fibroblasts remained attached without obvious change in morphology, indicating that fibroblasts are insensitive to ATP at the concentration tested (Fig. 4-7).
We also compared the sensitivity of SCs and astrocytes to high concentration of ATP. ATP (5 mM) was added to the co-culture of GFP transduced SCs and astrocytes (at 3:1 ratio). As seen in previous experiments, SCs started to round up within 15 min, while no obvious morphological change was observed in astrocytes (Fig. 4-8). At the current study using an antibody (rabbit anti-P2X7; Alamone Labs, Jerusalem, Israel) that recognizes the C-terminal part of P2X7R (Sim et al., 2004), no P2X7R-ir were observed on astrocytes from Wistar rats, and they were insensitive to high concentration of ATP. However, P2X7R was reported on mouse astrocytes (Duan et al., 2003; Nagasawa et al., 2009) and Sprague Dawley rat astrocytes (Panenka et al., 2001) in previous studies, which may be due to the different sources of astrocytes or the different antibody used (rabbit anti-P2X7, Chemicon) (Duan et al., 2003) that lack of specificity. It has been reported that P2X7R protein expression was much lower in rat than mouse astrocytes (Nagasawa et al., 2009).
Fig. 4-8 ATP induces Schwann cells death with no obvious effect on astrocytes in Schwann cell and astrocyte co-culture.

Phase contrast images show morphological changes and detachment of SCs in co-culture with astrocytes before (A) and after exposure to 5 mM ATP at 30 min (B) or at 2 h 30 min (C). (D) and (E) are images taken from the same field showing phase contrast (D) and fluorescence (E, green for SCs transduced with LV/GFP). The rounding of SCs and formation of varicosities on the processes of SCs (arrowheads in D and E) while astrocytes (arrows in D) remained the same morphology after exposure to 5 mM ATP at 100 min. Scale bar = 100 µm in A-C and 25 µm in D-E.

(4) P2X7R is responsible for ATP induced Schwann cell death

To test whether blocking P2X7R can rescue the SC from ATP induced death, we applied oxATP, an irreversible and slow P2X7R antagonist which requires 1 to 2 h incubation to block the functional activation of P2X7R (Di Virgilio, 2003) to the cultured SCs before the exposure ATP. oxATP was added to one group of culture dishes to a final concentration of 0.35 mM. After incubation for 2 h, oxATP treated and untreated cells (as control) were exposed to 3, 4, 5 mM ATP in the CO2 incubator for 1 h. By observation directly under a microscope, it was found that cells treated with oxATP remained the same while most of cells without oxATP treatment rounded or detached after exposure to ATP. SCs were then dissociated from the culture dishes and processed with Annexin V Apoptosis Assay kit using flow cytometry. It was shown that the percentage of live cells without/with oxATP treatment were 73.9% vs. 97.1%, 63.2% vs. 92.3%, and 52.3% vs.
93.6% at 3, 4, 5 mM ATP respectively. oxATP pretreatment completely rescued SCs from ATP induced cells death \( (p < 0.01, \text{Fig. 4-9}) \) and the percentage of live cells was similar to that of untreated cells in culture medium (92.0%).

**Fig. 4-9** Blockade of ATP induced Schwann cell death with the P2X7R antagonist oxATP *in vitro*. SCs were pretreated with 0.35 mM oxATP for 2 h before exposure to different concentrations of ATP. Untreated SCs were used as control. Flow cytometry apoptosis assay was performed 1 h after exposure to ATP. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) compared with control. ++\( p < 0.01 \) compared with the corresponding untreated group exposed to the same concentration of ATP. Experiments were repeated three times.

### 4.3.4 PSA partially rescues Schwann cell death induced by ATP *in vitro.*

We also found that PSA expression on SCs could partially protect SC from ATP induced cell death *in vitro* (Fig. 4-10). PSA expression on cells has been reported to reduce the cell attachment (Fujimoto et al., 2001). To exclude this potential effect that might cause the different sensitivity of PST/SCs or GFP/SCs to ATP toxicity, PST/SCs and GFP/SCs were first dissociated from the culture dishes and resuspended in 4 ml medium. Cell suspension was aliquoted into 1 ml to four 15 ml tubes. ATP of different concentration (0, 3, 4, 5 mM) was added into the tubes with cell suspension repetitively. The tubes were
incubated in the CO₂ incubator for 1 h with caps loosened. The tubes were gently shaken a few times to expose the cells to ATP evenly. The cell suspensions were then centrifuged at 800 rpm for 6 min and resuspend in 400 µl Annexin V Binding Buffer (BD). Cells suspension were incubated with 2 µl AnnexinV-Fluor 647 (BioLendeng) at RT for 15 min, then 5 µg/ml (final concentration) viability dye propidium iodide was added. The samples were processed immediately analysed with flow cytometry. The percentage of live cells in GFP/SCs and PST/SCs groups were 57.8% vs. 75.4%, 51.1% vs. 65.3% and 44.3% vs. 61.1% respectively after exposure to 3, 4 and 5 mM ATP for 1 h. There were significantly more live cells in PST/SCs group compared with GFP/SCs group at all three ATP concentrations ($p < 0.05$ or $p < 0.01$). As to whether such protective effect of PSA against ATP induced cell death reflects the improved survival of PSA-expressing SCs in spinal cord, further studies need to be carried out.

![Fig. 4-10 PSA partially protects Schwann cells from ATP induced death in vitro.](image)

Flow cytometry apoptosis assay was performed on PST/SCs and GFP/SCs. Graph shows the percentage of live cell after SCs were exposed to 3, 4 and 5 mM ATP for 1 h. Significantly more PST/SCs survived compared with GFP/SCs. *$p < 0.05$, **$p < 0.01$. Experiments were repeated three times.

### 4.3.5 Prevention of the death of transplanted Schwann cells with a P2X7R antagonist.

To test whether blockade of P2X7R can improve the survival of transplanted SCs, we
exploited the irreversible blockade property of oxATP. After the irreversible blockade of P2X7R in SCs, new P2X7Rs will be synthesized and transported to the cell membrane. First we studied the time window for SCs to become responsive again to ATP induced cell death after blockade of P2X7Rs with oxATP.

(1) *Recovery of blockade effect of oxATP*

oxATP (0.35 mM) was added to the SC culture medium. After incubation for 2 h, oxATP was washed out and fresh medium was replaced. At 3, 4, and 6 h after the wash, 3 or 4 mM ATP was added to the SC culture to test the recovery of SC response to ATP. By direct observation under a light microscope, it was found that between 4 and 6 h after oxATP washout, SCs started to be responsive to ATP again (Table. 4-1). This 4 h window may be enough to offer certain degree of protection against ATP induced SC death after transplantation as ATP release occurs instantly at the site of transplantation and may last for a few hours.

Table. 4-1 Recovery from the blockade effect of oxATP

<table>
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<tr>
<th>Time</th>
<th>oxATP 3 h washout % of rounded cells</th>
<th>oxATP 4 h washout % of rounded cells</th>
<th>oxATP 6 h washout % of rounded cells</th>
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<td>3 mM ATP</td>
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(2) *oxATP treatment increases the survival of transplanted Schwann cells*

Next we investigated whether blockade of P2X7R in SCs with oxATP could improve
their survival after transplantation into spinal cords. SCs from P2 pups of Wistar rat were transduced with LV/GFP for easy identification. One group of cells (100,000 in 1 µl) was treated with 0.35 mM oxATP for 2 h while the same number of untreated cells from the same batch was used as control. Both groups of cells were harvested at the same time and transplanted into either side of the spinal cord dorsal columns of adult Wistar rat (Fig. 4-11, n = 4).

Fig. 4-11 Diagram illustrates transplantation of GFP labelled Schwann cells with/without oxATP treatment into either side of dorsal column of rat T8 spinal cord.

One week later, animals were sacrificed and the areas occupied by GFP+ SCs in the spinal cord sections were measured using ImageJ. 38.2 ± 2.8% more oxATP treated SCs survived than the untreated SCs after transplantation (Fig. 4-12), indicating that blocking P2X7R in SCs can improve their survival after transplantation.
4.3.6 P2X7R activation induces neural stem cell death

It has been shown that P2X7R expressed in hematopoietic stem cells/progenitor cells and ATP could induce apoptosis in these cells (Yoon et al., 2007). More recently, it has been reported that P2X7R activation can mediate NPC death in vitro (Delarasse et al., 2009). This result may have significant implication for researchers using NSCs for transplantation as NSCs also undergo cell death after being transplanted into CNS although the survived cells can proliferate in the host tissue. To confirm this result, we did some preliminary experiments on NSCs from P7 pups of C57BL/6J mice. Immunocytochemistry study showed that P2X7Rs were widely expressed on NSCs (Fig. 4-13). Exposure of NSCs to 5 mM ATP induced significant morphological changes of NSCs similar to that of SCs observed under light microscope in vitro (Fig. 4-14), confirming that high concentration of ATP can also induce NSC death.

Fig. 4-12 oxATP pretreatment increases the survival of transplanted Schwann cells. (A) GFP labelled SCs transplanted into spinal cord. Right panel: pretreated with oxATP; left panel: without oxATP pretreatment. Dashed line indicates midline. (B) Quantification of the survived SCs 1 week after transplantation. n = 4, *p < 0.05.
Fig. 4-13 Expression of P2X7Rs on cultured neural stem cells. NSCs identified with a stem cell marker nestin stained with an anti-P2X7R antibody. Scale bar = 25 µM.

Fig. 4-14 ATP induces neural stem cells death in vitro. Phase contrast images show NSCs in culture before (A) and after exposure to 5 mM ATP at 15 min (B), 40 min (C), or 60 min (D). NSCs displayed similar response to 5 mM ATP as that of SCs, i.e., cells rounded, detached, shrank, or broken up over the experimental period.
4.4 Discussion

Much research has been performed to investigate the potential factors involved in cell death after transplantation. In the last few years we have been investigating whether over-expression of PSA on SCs can promote their survival and migration after transplantation into the spinal cord. However, compared with the enhanced migration ability, the survival of PSA-expressing SCs is still far from satisfactory, indicating additional factors may be unidentified.

In this study, we first tested the effect of glutamate on the survival of SCs. Unlike the excitotoxicity of glutamate to neurons, glutamate up to 1 mM did not induce SCs death. Glutamate plays important roles in axon-SC signalling. It has been shown that glutamate or a glutamate-like substance is released from axons during excitation to activate a kainate receptor on the SCs (Lieberman, 1991). Moreover, AMPA receptor subunits: GluR2/R3 and GluR4 have been detected on SCs (Dememes et al., 1995). But, NMDA receptors have not been proved to be located on rat SCs, which may explain the insensitivity of SCs to high dose of glutamate.

During the SCs harvesting process for transplantation, the original culture medium which contains serum and growth factor cocktails was replaced with DMEM only as the suspension medium. Serum and growth factor withdrawal may not exactly mimic the environment SCs encountered after being transplanted into spinal cord. However, this instant shock may still affect the survival of transplanted cells. Indeed, in this study, we found that the prolonged withdrawal of serum and growth factors induced significant cell death in vitro. In our study, over-expression PSA on SCs can partially protected SCs from serum and growth factor withdrawal induced cell death. The underlying mechanism for such phenomenon is still unknown and needs to be explored in future studies.

An important discovery in the current study is that high concentration of ATP can induce SC death in vitro. The evidence provided in the current study indicates that P2X7R is the subtype of P2X receptors that mediates the SC death. One line of evidence is from the concentration of ATP used. No obvious cell death was detected with ATP concentration lower than 1 mM, as P2X7R is the only subtype of P2X receptors that is activated with ATP in mM range. Another line of evidence is that P2X7R agonist BzATP
was also potent in inducing SC death. The third line of evidence is that P2X7R antagonist oxATP can block the SC death induced by ATP. We also performed a preliminary experiment by exposing SCs from P2X7R knockout and wild-type mice to 4 mM ATP and subjected the cells to flow cytometry. SCs from P2X7R knockout mice showed no obvious cell death (95% live cells), while only 4% SCs from the wild-type mice were alive (no graph is presented as the experiment needs to be repeated). The functional data were further supported by the evidence that P2X7Rs are expressed in cultured SCs and NSCs, but not in fibroblasts and astrocytes which were resistant to ATP induced cell death. Moreover, deletion of P2X7R gene abolished the ATP induced SC death.

The above in vitro evidences clearly demonstrate that high concentration of ATP can induce SC death. It is tempting to speculate that ATP may contribute to the death of the transplanted SCs in the spinal cord. One crucial question is that whether ATP released during transplantation procedure will reach concentration high enough to induce SC death. It has already been known that concentrations of ATP in cells are in the range of 1-10 mM (Beis and Newsholme, 1975). Upon cell breakage after injury, intracellular ATP will be released and the local concentration of ATP could be in mM range. It has been reported that mechanical stimulation can lead to release of ATP from astrocytes (Newman, 2001). Sustained high-level ATP release at spinal cord injury site was reported to last for 6 h (Wang et al., 2004). Cell transplantation procedure, although it has been done very carefully to minimize the damage to the host tissue, certain injury is unavoidable due to penetration of injection needle. Also the space occupied by transplanted cells will put pressure on the surrounding host tissues which may trigger the release of ATP from astrocytes. Therefore, it is highly possible that released ATP at the transplantation site may reach mM range and induce the death of transplanted SCs.

By using the irreversible P2X7R antagonist oxATP to block the P2X7R on SCs before transplantation, we did observe significantly improved survival of SCs in comparison with untreated SCs. However, the application of oxATP to improve the survival of transplanted cells may not be an ideal approach since oxATP has many other targets like ATPases in the cells apart from P2X7R and is cytotoxic to cells (Craighead et al., 2001). We did observe that higher concentration of oxATP such as 0.5 mM could induce a certain degree of cell death (data not presented). At 0.35 mM, oxATP induced
SC death was not statistically significant although the percentage of live cells was lower than the control group. Another interesting phenomenon we observed but didn't have time to explore further is that ATP seems to have certain protective effect against the toxicity of oxATP on SCs, i.e., the percentage of live cells in the group treated with oxATP and ATP was higher than that the group treated with oxATP only. One potential explanation is that after the blockade of P2X7Rs, ATP may activate other subtypes of P2 receptors, especially the metabotropic P2Y receptor subtypes. It is also possible that adenosine derived from ATP may also exert protective effect via adenosine receptors on SCs (Stevens et al., 2004). Another reason that oxATP is not an ideal P2X7R blocker for the in vivo study is that, although irreversible, newly synthesized P2X7Rs will make SCs sensitive to ATP again 4 h after removal of oxATP. The sensitivity returned to oxATP pre-treatment level after 16 h. Therefore, other approaches which are more specific at targeting P2X7R and have longer effects need to be developed. One potential method is to use small interfering RNA (siRNA) to knock down P2X7Rs in SCs and then study their survival after transplantation into spinal cord. Several publications have reported successful knockdown of P2X7R with siRNA (Chen et al., 2008; Cesaro et al., 2010).

P2X7Rs have also been reported on several types of cells in the CNS, mostly on microglia (Di Virgilio et al., 1999). This receptor has been reported to participate in the processing and release of cytokines such as IL-1β, and in the initiation of cell death via both apoptotic and necrotic pathways (Schneider et al., 2006). In the CNS, P2X7R has been implicated in many pathological processes including neuroinflammation (Le Feuvre et al., 2002; Sperlagh et al., 2006; Skaper et al., 2010). P2X7R mediated release of inflammatory factors at the injury site may also contribute to the death of transplanted cells. In the normal rodent brain, P2X7R expression in astrocytes is generally quite low, but quickly up-regulated in response to brain injury or pro-inflammatory stimulation in cell culture conditions (Franke et al., 2004; Narcisse et al., 2005). In astrocytes, P2X7R activation can potentiate pro-inflammatory singalling, as it enhances IL-1β-induced activation of NF-κB and activator protein 1, leading to increased production of nitric oxide as well as increased production of the chemokines monocyte chemoattractant protein-1 and IL-8 (John et al., 2001; Panenka et al., 2001). Such processes may lead to more inflammatory factor release via the activation of P2X7R. It is likely that more cell
death will occur after cells being transplanted into the lesioned spinal cord. It was reported that intravenous administration of Brilliant Blue G (BBG), a commonly used food additive, and also a selective P2X7R antagonist, significantly reduced spinal cord anatomic damage, and improved motor recovery after spinal cord injury (Peng et al., 2009). BBG treatment also directly reduced local activation of astrocytes and microglia, and neutrophil infiltration. We have planned to administrate BBG to rats before the transplantation of SCs to see such treatment would also be able to enhance the survival of transplanted SCs. If the treatment is effective, further enhancement of survival may be achieved by combining with knockdown of P2X7Rs on SC.

The results obtained in the current study indicate that blockade P2X7Rs in SCs may promote their survival after transplantation and improve function recovery. Moreover, in our preliminary study, it confirmed that NSCs, a potential cell source for treating neurodegeneration diseases like Parkinson’s disease and Huntington’s disease (Dunnett et al., 2001; Wright and Barker, 2007), also express high-level of P2X7Rs. Furthermore, high concentration of ATP also induced NSC death. Targeting P2X7R may also be an effective approach to improve the survival of NSCs/NPCs after transplantation. Therefore, the current work on P2X7R induced SC death may have much bigger impact in the cell therapy field.

Another interesting finding is that PSA can also partly protect SC from ATP induced cell death. It may also contribute to the better survival of grafted PST/SCs. However, we are unable to provide any explanation to the phenomenon like the protection of PSA against serum and growth factor withdrawal. One potential mechanism is that PSA may prevent the aggregation of P2X7R subunits to form the pores that leads to cell death. Further experiments need to be carried out to explore the underlying mechanisms.
Chapter 5
Migration of transplanted Schwann cells in uninjured and injured spinal cord

5.1 Abstract
Lack of migration is one of main drawbacks that limit the success in using SC transplantation for neural repair after neurotrauma. In order to promote the migratory capability of the transplanted SCs, we genetically modified SCs to over-express PSA to reduce the adhesion of SCs. PST/SCs or GFP/SCs (as control) were first injected into uninjured rat spinal cord and it was found that expression of PSA on SCs did not enhance the motility of transplanted SCs in uninjured spinal cord. However, when SCs were transplanted 2.5 mm caudal to a spinal cord lesion site, in contrast to the GFP/SCs which only spread a short distance towards the lesion site, PST/SCs showed enhanced migration toward the lesion site along the degenerating dorsal column, and some PST/SCs were found within the lesion cavity 3 weeks after transplantation. Furthermore, when transplantation of PST/SCs was combined with LV/PST-GFP injection around the lesion site to induce the expression of PSA in the host spinal cord, more PST/SCs penetrated the glial scar and migrated into the lesion cavity. Similarly, GFP/SCs transplantation combined with LV/PST-GFP injection demonstrated increased migration of GFP/SCs along the PSA⁺ pathway toward the lesion site, but migration distance was shorter than that of PST/SCs combined with LV/PST-GFP injection. These results demonstrate that: (1) some molecules released from the spinal cord injury site are chemotactic factors for SCs; (2) PSA expression on SCs enhanced their migration towards lesion site; (3) PSA expression around the lesion site can render the scar more permissive and further facilitate SC migration and penetration into the lesion site.

5.2 Aims
In this study, we aimed to study: (1) whether PSA expression on SCs can enhance their migration in uninjured spinal cord or (2) in injured spinal cord; (3) whether PSA expression on the spinal cells around the lesion site can further enhance PSA
expressing-SCs migration and infiltrate to the lesion site.

5.3 Results

5.3.1 Migration of Schwann cells in uninjured spinal cord.
To investigate the migration of SCs in uninjured spinal cord, 24 Wistar rats from each group received either GFP/SCs or PST/SCs transplantation with ciclosporin injection daily to reduce the effect of immune rejection. Animals were killed 1, 7, and 30 days after transplantation. It was found that transplanted SCs concentrated at the injection site, with some cells spreading a short distance rostrally and caudally along the axis of the spinal cord 1 day, 7 days, and 30 days after transplantation in both PST/SCs and GFP/SCs groups. Distances of 20 cells spreading the furthest from the epicenter of the transplants on three selected sections from each animal was measured. The migration distance for GFP/SCs and PST/SCs group were 768 ± 96 μm vs. 807 ± 109 μm at 1 day and 731 ± 162 μm vs. 828 ± 111 μm at 7 days post transplantation respectively. No marked difference in the distribution of cells in both groups between 1 day and 7 days post transplantation was found, which indicates SCs implanted into uninjured spinal cord do not migrate. At 30 days post transplantation migration distances in both groups showed tendency of decreasing (580 ± 48 μm vs. 644 ± 106 μm for GFP/SCs and PST/SCs respectively), although the decrease was not statistically significant compared with those at 1 and 7 days. The decrease in migration distances may be explained by cell death over time.
Fig. 5-1 Migration of Schwann cells in uninjured spinal cord.
Distance of 20 cells spreading furthest from the epicentre of the transplants on three selected sections from each animal was measured with ImageJ programme to assess the migration of SCs. There is no difference in migration distances between PST/SCs and GFP/SCs at 1 day or 7 days after transplantation. Decreased migration distances were observed in both groups at 30 days after transplantation. n = 8.

5.3.2 Migration and infiltration of Schwann cells in injured spinal cord.
To study the migration of transplanted SCs in injured spinal cord, PST/SCs or GFP/SCs (n = 6 per group) were injected 2.5 mm caudal to the lesion site of a crush injury at T8 level. Animals were treated with ciclosporin daily. Three weeks after transplantation, significantly more PST/SCs were seen to migrate from the centre of the grafts towards the lesion site than GFP/SCs (1440 ± 211 vs. 696 ± 111 µm, p < 0.05, Fig. 5-2B-C). Most of the migrating PST/SCs were located close to the glial scar around the lesion site, while GFP/SCs were found drifting a short distance towards the lesion site (Fig. 5-2A, C). The results indicate that some molecules released from the lesion site act as chemotactic factors for SC migration and PST/SCs are more mobile than GFP/SCs. Density of GFP+ SCs within the lesion cavity defined by lack of GFAP fluorescence was measured. In two out of six animals, a large number of PST/SCs migrated into the lesion cavity, while in GFP/SCs group, only a very small proportion of the GFP/SCs migrated into the lesion cavity in one out of six animals (Fig. 5-3).
Fig. 5-2 Migration of Schwann cells transplanted caudal to the lesion cavity of spinal cord.
Consecutive parasagittal sections showing GFP/SCs (A) and PST/SCs (B) migrating toward the lesion site. (C) Distances of SC migration from the injection site towards the lesion site 3 weeks after transplantation. *p < 0.05. Axes indicate the orientation of the spinal cord. Scale bar = 100 µm. n = 6.
Fig. 5-3 Infiltration of transplanted Schwann cells into the injury site of the spinal cord.
Quantification of transplanted SCs migrated into the lesion site 3 weeks after transplantation. Integrated density of GFP-ir within the lesion cavity was measured using imageJ. Each dot represents an individual animal.

We then explored whether engineered expression of PSA in the glial scar around the lesion site would further enhance the migration and infiltration of PST/SCs into the lesion cavity. Four groups of rats received four different combinations of treatments (Table 5-1, Fig. 5-4). Briefly, GFP/SCs or PST/SCs were transplanted 2.5 mm caudal to the lesion site of spinal cord, immediately followed with LV/mCherry or LV/PST-GFP injection around the lesion site (n = 5-7). It was found in the group of GFP/SCs transplantation plus LV/mCherry injection, the migration distance of GFP/SCs was similar to that in the group without mCherry expression. Likewise, expression of mCherry also did not affect the migration of PST/SCs towards the lesion. In contrast, expression of PSA in spinal tissue around the lesion site significantly promoted the migration of both GFP/SCs and PST/SCs towards the lesion (Fig. 5-5B, D, F). In the group of PST/SCs plus LV/PST-GFP injection, PST/SCs penetrated the glial scar and migrated into the lesion cavity 3 weeks after transplantation in 4 out 5 rats (Fig. 5-5G); while SC migration into lesion cavity was only observed in 1 out 7 rats in the GFP/SCs plus LV/PST-GFP injection group, and 2 out of 5 in PST/SCs plus LV/mCherry injection group (Fig. 5-5G). There was no transplanted SC in the lesion cavity in the group of GFP/SCs plus LV/mCherry injection.
Since CSPGs is one of the main inhibitory molecules secreted by astrocytes, we also checked whether LV/PST-GFP injection around the lesion site would alter the CSPGs expression. High level of PSA expression was found in LV/PST-GFP injection group, however, no marked difference was found in CSPGs expression levels in groups with or without LV/PST-GFP injection (Fig. 5-6).

![Diagram illustrating the surgery used to assess the migration of SCs](image)

**Fig. 5-4 Diagram illustrates the surgery used to assess the migration of SCs**

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<tr>
<th>Table. 5-1 Schwann cell transplantation plus LV injection</th>
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Fig. 5-5 Migration of Schwann cells transplanted caudal to the injury site of spinal cord.

SCs were injected 2.5 mm caudal to the lesion and the animals were sacrificed 3 weeks after transplantation. Migration of transplanted GFP/SCs combined with LV/mCherry (A) or LV/PST-GFP (B) injection around the lesion site. Migration of transplanted PST/SCs combined with LV/mCherry (C) or LV/PST-GFP (D) injection around the lesion site. (E) Adjacent section to (D) showing PST/SCs within the lesion cavity. Arrows point to the injection sites. (F) Graph illustrating the distance of SC migration from the injection cavity towards the lesion site 3 weeks after transplantation. (G) Quantification of transplanted SCs migrated into the lesion site 3 weeks after transplantation. Integrated density of GFP-ir within the lesion cavity was measured using imageJ. Each dot represents an individual animal. *p < 0.05. Axes indicate the orientation of the spinal cord. Scale bars = 250 µm for A-D, 100 µm for E.
Fig. 5-6 PSA expression does not alter CSPGs expression.
Expression of PSA (blue) and CSPGs (red) around the lesion site of spinal cord with or without LV/PST-GFP injection. No marked difference was found in CSPGs expression level in groups with or without LV/PST-GFP injection 3 weeks after surgery. Scale bar = 100 µm.

5.3.3 Remyelination of axons by PST/SCs
The potential of the grafted SCs to remyelinate axons was evaluated by detection of GFP⁺/P0⁺ myelin internodes. Nine weeks after transplantation of SCs directly into the lesion site of a hemi-transection injured T8 spinal cord, strong P0-ir was found within the
transplants (Fig. 5-7B-C) and the pattern of distribution of P0-ir closely matched the GFP fluorescence from transplanted SCs. Confocal photomicrograph confirmed that many PST/SCs around neurofilament (NF)$^+$ axons were P0$^+$ (arrowheads in Fig. 5-7D). Some P0$^+$ internodes were GFP negative indicating that they originated from the host (Fig. 5-7C-D).

**Fig. 5-7 Myelination of axons by PST/SCs.**
LV/PST-GFP and LV/GFP co-transduced SCs (A) grafts stained with P0 (B) 9 weeks after transplantation. (C) is the merged image of (A) and (B). Confocal photomicrograph shows PST/SCs (green) stained with P0 (red) and NF (blue) antibodies. Arrowheads point to the PST/SCs co-expressing P0 protein around NF$^+$ axons. Scale bar for A-C = 100 µm; scale bar for D = 20 µm.
5.4 Discussion

PSA has been shown to play an important role in progenitor cell migration during development (Franceschini et al., 2004; Zhang et al., 2008). The underlying mechanisms are unclear. It was suggested that the large negatively charged PSA chain may act as a spacer that reduces adhesion force between cells, thereby, allowing dynamic changes in membrane contacts and promoting morphological plasticity and cell movement (Rutishauser et al., 1988). *In vitro* PSA-expressing SCs demonstrated increased migration potential when they were injected into a slice culture of postnatal brain (Lavdas et al., 2006). However, when the same STX-transduced SCs (STX/SCs) were transplanted 0.5 mm rostrally to the lesion site of mouse spinal cord STX/SCs did not show enhanced migration capability (Papastefanaki et al., 2007). This observation contradicts our result that PST/SCs show enhanced migration towards the lesion site. The lack of enhanced migration of STX/SCs in Papastefanaski’s study was explained by the early down-regulation of PSA on the transplanted SCs. In the current study, we also found PSA down-regulation over the periods after transplantation (See chapter 3, Fig. 3-4) and PSA-ir was hardly detected on PST/SCs nine weeks after transplantation. However, compared with STX/SCs, the expression of PSA on transplanted SCs driven by PST lasted for much longer periods, which led to delayed myelination by PST/SCs (unpublished observation). Our results agree with the report that prolonged expression of PSA on implanted neural precursors driven by PST-GFP fusion protein is associated with delayed myelination (Franceschini et al., 2004). Down-regulation of PSA on oligodendrocyte precursor cells has been reported to be necessary for these cells to differentiate into myelin-forming cells (Fewou et al., 2007). Although the mechanism regulating PSA expression remains obscure, it might result from post-transcriptional regulation triggered by SC differentiation after grafting. As a consequence, over-expression of PSA in SCs did not impair their myelinating potential. Indeed, we found strong P0-ir presented within the PST/SCs grafts nine weeks after transplantation; some P0+ internodes were GFP-negative indicating that they originated from the host. It is consistent with the previous report that endogenous SCs recruited from the periphery to the lesion site through the dorsal root entry zones contribute to remyelination (Beattie et al., 1997). It appears that STX- and PST-transduced SCs show differential response to the
myelinating signals when they were transplanted into the spinal cord. This may not be surprising, since STX is mainly expressed in early developmental stage, while PST is expressed in the postnatal and persists in adult nervous system, and they may respond differently to the different environment signals (Ong et al., 1998). Furthermore, it was also reported that differentiation of PSA+ neuroblastoma cell lines induced by retinoic acid led to decrease in STX mRNA level, in contrast, it resulted in a dramatic increase of PST mRNA level (Seidenfaden and Hildebrandt, 2001). In another study, different levels of induced expression of PSA in chick retina by retroviral delivery of PST and STX has also been reported, i.e., PST induced PSA expression in retina resulted in severe localized alterations in retinal morphogenesis, while STX did not induce PSA expression or affect morphogenesis (Canger and Rutishauser, 2004).

Our current data showed over-expression of PSA on SCs did not have effect on their migration ability when they were transplanted into uninjured spinal cord, but facilitated the migration of the cells along the degenerating dorsal column toward the lesion site, indicating that some molecules released from the injury site act as chemotactic factors to attract the transplanted SCs to the injury site. This result is consistent with previous studies. It was shown that oligodendrocyte progenitor migration in response to injury of glial monolayers required PSA on the progenitors (Barral-Moran et al., 2003). It was also found that PSA was necessary for directional migration of oligodendrocyte progenitors toward a gradient of PDGF in vitro and removal of PSA severely impaired PDGF gradient mediated directional migration while the directional migration of progenitors toward basic fibroblast factor source was not affected by the removal of PSA (Zhang et al., 2004). Furthermore, PSA has been shown modify the ability of cells to sense accurately to other growth factor gradients such as BDNF (Muller et al., 2000; Vutskits et al., 2001) and CNTF (Vutskits et al., 2003). These results suggest that PSA expression plays an instructive role in the guidance of migrating cells to the molecular cues released from the lesion site. In Papastefanaski’s study both the STX/SCs and the control alkaline phosphatase transduced GFP SCs were injected 0.5 mm rostral to the compression lesion site, which was much closer to the lesion site than in our study (2.5 mm). In 4 weeks, SCs from both groups populated the lesion site, which also indicates the molecules released from the lesion have strong attraction to the transplanted SCs. In a
recent study it was reported that STX transduced macaque SCs did show enhanced migration to a demyelinated site in the spinal cord of nude mice when STX/SCs were transplanted 2 mm from the lesion site, and the STX/SCs were observed to migrate along laminin$^+$ structures in the spinal cord (Bachelin et al., 2010). The requirement for the presence of chemotactic cues for the migration of transplanted PSA-expressing SCs may explain why they did not migrate when transplanted into uninjured spinal cord. In injured spinal cord, PSA may facilitate SCs migration by reducing cell adhesion and/or priming the cells to respond to chemotactic factors.

The cellular mechanism by which the PSA-expressing scar tissue permitted the SCs migration and penetration remains unclear. It is difficult to detect the subtle change of the scar tissue by over-expression of PSA. The action of PSA may occur at the level of contact dependent cell signaling and buffer the detrimental effects of growth inhibitory molecules present in the scar tissue, such as CSPGs. However, it was found that PSA expression does not alter CSPGs after injury which is consistent with previous study (El Maarouf et al., 2006).

The current study showed that when PST/SCs transplantation was combined with induced expression of PSA in the spinal cord close to the caudal lesion site more PST/SCs were observed migrating into the lesion cavity. Similarly, induced expression of PSA in the spinal cord also enhanced the migration of GFP/SCs, but to a lesser extent compared with PST/SCs. Our previous study showed that engineered expression of PSA in the dorsal root entry zone and spinal cord increased migration of endogenous SCs along the PSA$^+$ pathway into the spinal cord (Zhang et al., 2007c). Likewise, engineered expression of PSA on astrocytes located along a path extending from the SVZ to a lesion near the cortical surface increased the recruitment of precursor cells along the path and into the injury site (El Maarouf et al., 2006). We have previously shown that expression of PSA on glial scar around spinal cord lesion site loosen the glial scar and rendered it permissive for axon growth (Zhang et al., 2007b). Taken together, it suggests that over-expression of PSA in the host tissues can create a more permissive environment and facilitate migrating cells penetrating into the lesion cavity.
Chapter 6
Interaction of Schwann cells with astrocytes in vivo and in vitro

6.1 Abstract
Transplantation studies of SCs in animal models have revealed several drawbacks such as causing stress response of host CNS tissues that limit the integration of transplanted SC (Lakatos et al., 2003a). In this study, we investigated whether expression PSA on SCs could modify their interaction with host tissues in vivo and interaction with astrocytes in vitro. In the in vivo experiments it was shown that the transplanted GFP/SCs formed a sharp boundary with host tissues and caused stress response of host tissues, demonstrated by increased expression of GFAP-ir and CSPGs-ir in the adjacent host tissues. In contrast, transplanted PST/SCs intermixed with the host cells and did not cause such stress responses of the host spinal cord. Using in vitro SCs/astrocytes confrontation assay we mimicked the interaction of the two types of cells in vivo and found that significantly more PST/SCs crossed a 300 µm line drawn along the SCs/astrocytes confrontation boundary (23.0 ± 1.6 cells in PST/SCs group vs. 5.6 ± 0.5 cells in GFP/SCs group, \( p < 0.001 \)). By measuring the areas of individual astrocytes at the boundary it was shown that GFP/SCs caused significant hypertrophy in comparison with PST/SCs (7843 ± 411 µm² in GFP/SCs group vs. 3635 ± 136 µm² in PST/SCs group, \( p < 0.001 \)). Significantly more astrocytes proliferated when they were co-cultured with GFP/SCs than with PST/SCs. There was no difference in astrocytes proliferation when they were exposed to conditioned medium from both groups. The results indicate that expression of PSA on SCs can modify the property of SCs and make them less hostile to astrocytes.

6.2 Aims
In this study, we addressed whether PST/SCs behaved differently from GFP/SCs when they interacted with astrocytes both in vivo and in vitro. We tested (1) the integration of PST/SCs or GFP/SCs with host tissues and the stress response of the spinal cells to the transplanted SCs; (2) the interaction of PST/SCs or GFP/SCs with astrocytes using confrontation assay and co-culture assay.
6.3 Results

6.3.1 CSPG expression on naïve astrocytes and Schwann cells

Since CSPGs are one family of the main inhibitory molecules secreted by astrocytes after spinal cord injury, we first detected and compared CSPG expression on naïve astrocytes and SCs in vitro. After permeabilization astrocytes were immunostained with antibodies against CSPG and GFAP, and SCs were immunostained with antibodies against CSPG and p75NTR. CSPG-ir was found to be localized in the cytoplasm in many GFAP+ astrocytes, while only detected in a few p75NTR+ SCs (Fig. 6-1).

![Fig. 6-1 Detection of CSPG expression in astrocytes and Schwann cells in vitro. CSPG expression was detected by immunostaining with CS-56 antibody in astrocytes (A) and SCs (B). High-level CSPG-ir was detected in many astrocytes (GFAP+, green in A) but was only present in a small population of SCs (p75NTR+, green in B). Scale bar = 100 µm.](image)

6.3.2 Response of host tissues to the transplanted Schwann cells

6.3.2.1 PSA expression in Schwann cells reduced stress response of neighbouring astrocytes in vivo.

To investigate the interaction of SCs grafts with host tissue, sections of spinal cord
containing the transplanted SCs were immunostained with antibodies against GFAP or CSPG. It was found that grafted GFP/SCs formed a sharp boundary with the host astrocytes (Fig. 6-2A) while PST/SCs intermixed well with the host astrocytes (Fig. 6-2B).

![Fig. 6-2 Interaction of transplanted Schwann cells with host astrocytes.](image)

Confocal images showing that transplanted GFP/SCs (A) formed a sharp boundary with the host GFAP⁺ astrocytes in the spinal cord while PST/SCs (B) integrated well with the host astrocytes. Scale bar = 50 µm.

Increased GFAP and CSPG expression has been regarded as a sign of astrocytic stress. In this study we found that transplanted GFP/SCs induced increased expression of GFAP-ir (Fig. 6-3A-C) and CSPGs-ir (Fig. 6-3G-I) in the neighbouring astrocytes close to the transplanted GFP/SCs. In contrast, much weaker GFAP-ir (Fig. 6-3D-F) and CSPGs-ir were observed close to the transplanted PST/SCs (Fig. 6-3J-L). The results indicate that PSA expression on SCs changed their properties and made them less hostile to the astrocytes.
Fig. 6-3 GFAP and CSPG immunoreactivities around the transplanted Schwann cells.

GFP/SCs (A) and PST/SCs (D) immunostained with a GFAP antibody (B, E) 7 days after being grafted into rat spinal cord. (C) and (F) are merged images of (A), (B), and (D), (E) respectively. GFP/SCs (G) and PST/SCs (J) immunostained with CS-56 antibody (H, K) 7 days after being grafted into rat spinal cord. (I) and (L) are merged images of (G), (H) and (J), (K), respectively. There is less GFAP-ir and CSPG-ir around PST/SCs. Scale bar = 100 µm.
6.3.3 Response of astrocytes to induced expression of PSA \textit{in vitro} \\
We have previously shown that PSA expression did not alter the level of CSPG expression in astrocytes transduced with LV/PST-GFP \textit{in vivo} (see Chapter 5, Fig.5-7). Proliferation is another index to evaluate astrocytic stress response. Here we tested whether PSA expression could change the proliferation of astrocyte \textit{in vitro}. Astrocytes were transduced with either LV/GFP or LV/PST-GFP. Cells were maintained in culture medium for a few days to achieve high level of transgene expression. BrdU (20 µM) was then added 16 h before the cells were fixed. It was found that PSA expression in astrocytes did not alter their proliferation (27.9 ± 2.0% vs. 28.8 ± 1.8% for LV/GFP and LV/PST-GFP transduced astrocytes respectively, Fig. 6-4).
Fig. 6-4 Expression of PSA in astrocytes did not alter the proliferation.

(A) Astrocytes transduced with LV/GFP stained with PSA. (B) Astrocytes transduced with LV/PST-GFP double stained for PSA (red) and GFAP (green). PSA-ir was detected in Golgi apparatus where PST-GFP was located when cells were permeabilized. (C1-C3) Astrocytes transduced with LV/GFP with BrdU staining. (D1-D3) Astrocytes transduced with LV/PST-GFP with BrdU staining. (E) Graph shows the percentage of BrdU⁺ nuclei of astrocytes transduced with LV/PST-GFP or LV/GFP. No difference was found between these two groups. Scale bar = 25 µm. Experiments were repeated three times.
6.3.4 Interaction of Schwann cells with astrocytes in vitro

6.3.4.1 Confrontation assays

Confrontation assays have been used to demonstrate that SCs are less capable of mingling with astrocytes and induce hypertrophy of astrocytes (Lakatos et al., 2000; Fairless et al., 2005; Santos-Silva et al., 2007). In this study, an in vitro confrontation assay was set up to investigate the interaction of PST/SCs with astrocytes in comparison with GFP/SCs. A strip containing 50,000 Schwann cells in 20 µL medium was set up opposing a parallel strip containing 50,000 astrocytes in 20 µL on a PLL-coated coverslip. Cells were allowed to attach for 30 min before washing with DMEM to remove non-attached cells. Cultures were then maintained in 10% DMEM-FBS over a period of 12–14 days, giving time for cells to make contact and interact. Cells were then fixed. Astrocytes were immunolabelled using a monoclonal anti-GFAP antibody and SCs using a polyclonal anti-p75NTR antibody. For quantification, a 300 µm line was drawn along the interface between astrocytes and SCs. The number of SCs that crossed the cell interface was counted and averaged over five randomly chosen fields on each coverslip (8 coverslips per group per experiment) and experiments were repeated three times. Here, we confirmed that GFP/SCs formed a distinct boundary with astrocytes when the two populations of cells came into contact with each other (Fig. 6-5A, C) and only a few SCs crossed into astrocyte territory (Fig. 6-5E). In contrast, no clear boundary was formed at the interface between PST/SCs and astrocytes (Fig. 6-5B, D). Significantly more PST/SCs crossed the line drawn at the interface of the two types of cells and intermingled with astrocytes (Fig. 6-5E). To assess the hypertrophy of astrocytes in contact with PST/SCs or GFP/SCs, the cytoplasmic areas of astrocytes (100 each) defined by GFAP-ir were measured using ImageJ and expressed in µm². It was found that GFP/SCs contact induced significantly higher level of astrocyte hypertrophy compared with PST/SCs (7843 ± 411 µm² vs. 3635 ± 136 µm² for GFP/SCs and PST/SCs group respectively, p < 0.001, Fig. 6-5F).
Fig. 6-5 Schwann cell and astrocyte confrontation assay in vitro.
Photomicrographs (A and C) showing that a distinctive boundary formed when GFP/SCs encountered astrocytes. Photomicrographs (B and D) showing that PST/SCs penetrated the astrocyte boundaries and populated the astrocytic domain. The yellow line illustrates a typical 300µm line drawn to quantify the number of cells that have crossed the boundary between the astrocytes and either PST/SCs or GFP/SCs. (E) Quantification of the numbers of SCs crossing the boundary line. (F) Quantification of the cytoplastic areas of the individual astrocytes along the boundary with GFP/SCs or PST/SCs as indicator for cell sizes. ***p < 0.001. Scale bars = 100 µm. Experiments were repeated three times.
6.3.4.2 Assessment of hypertrophy and proliferation of astrocytes in astrocyte & Schwann cell co-culture

To assess whether astrocytes require direct contact with SCs for the induction of stress response, co-cultures were set up to further study the interaction of PST/SCs with astrocytes in comparison with GFP/SCs. Primary cultures were generally maintained for 2 – 4 weeks before setting up the assays. Astrocytes and SCs were mixed at a ratio of 3:1 and 2 × 10^5 cells in total were plated on PLL-coated 10 cm dish in DMEM- 10%FBS. These cultures were maintained for 10-14 days. BrdU (20 µM) was added 16 h before fixation of cells to assess the proliferation of astrocytes. To analyze the proliferation of astrocytes, 10 randomly selected fields of view per coverslip under a 20× objective lens of a Leica microscope were captured for counting. Six coverslips were used per experiment and the experiment was repeated three times. Astrocytes were identified with GFAP antibody. The data are expressed as the ratio of double GFAP^+/BrdU^+ cells over double GFAP^+/DAPI^+ cells for the level of proliferation of astrocytes. It was found that significantly more astrocytes proliferated in the GFP/SCs and astrocytes co-culture than in the PST/SCs and astrocytes co-culture (22.6 ± 1.2% vs. 17.8 ± 1.0%, p < 0.01, Fig. 6-6).
Fig. 6-6 BrdU incorporation assay for astrocytes in cultured with Schwann cells. 
(A-E) GFP/SCs and astrocytes co-culture with BrdU staining. (F-G) PST/SCs and astrocytes co-culture with BrdU staining. (K) Percentage of BrdU$^+$ nuclei of astrocytes. Significantly more astrocytes proliferated in the GFP/SCs and astrocytes co-culture than in the PST/SCs and astrocytes co-culture. Scale bar = 100 µm. **p < 0.01. Experiments were repeated three times.

6.3.4.3 Assessment of hypertrophy and proliferation of astrocytes induced by Schwann cell conditioned medium.

To assess whether released factors from SCs are responsible for the induction of astrocytic stress, we performed astrocyte culture with conditioned medium collected from GFP/SCs or PST/SCs. Briefly, when cultures of purified SCs were confluent (maintained in 60 mm culture dish in vitro for 1-2 weeks), they were rinsed twice with PBS and 4 ml of DMEM-10% FBS without added growth factors. Cultures were maintained for two
more days before medium collection. Collected media were centrifuged to remove cell debris, and filtrated through a 0.2 µm filter (Millipore, Hertfordshire, UK). SC conditioned medium (SCM) was added to astrocyte culture at a 1:1 or 1:5 ratio with DMEM-10% FBS. The culture was maintained in SCM for 2 days. BrdU (20 µM) was added 16 h before fixation with 4% PFA and the cells were immunostained with antibodies against BrdU in order to analyze proliferation of astrocytes. It was found the proliferation level of astrocyte in GFP/SCs conditioned medium and PST/SCs conditioned medium were 25.7 ± 3.0% vs. 22.8 ± 3.3% (1:1 dilution in DMEM-10% FBS), 26.8 ± 2.4% vs. 22.0 ± 4.4% (1:5 dilution in DMEM-10%FBS, p > 0.05, Fig. 6-7). There was no significant difference between the GFP and PST groups. Also no difference between the 1:1 and 1:5 dilution groups. The results indicate that stress response of astrocyte induced by SCs is due to direct contact rather than secreted factors from SCs.

Fig. 6-7 Effect of Schwann cell conditioned medium on the proliferation of astrocytes.
SCM was diluted in DMEM-10% FBS at 1:1, and 1:5, and BrdU incorporation into astrocytes was measured. There is no significant difference between GFP/SCs and PST/SCs conditioned medium groups. Experiments were repeated three times.
6.3.5 PSA expression on olfactory ensheathing cells

In previous studies, OECs have been reported to induce much less astrocyte stress compared with SCs and mingle well with host tissues (Lakatos et al., 2000; Lakatos et al., 2003a; Santos-Silva et al., 2007). In this study, we found that PSA-expressing SCs behaved in the similar way as OECs when they interacted with astrocytes. OECs were classified into two cell types based on morphology and antigenic phenotype. OECs with astrocyte-like morphology express both GFAP and PSA (identified with a monoclonal IgM antibody) (Franceschini and Barnett, 1996). We postulate that PSA might be the key molecule that makes SCs to gain those OEC-like properties. In a preliminary study, we also examined PSA expression on OECs. OECs were obtained from Professor Geoffrey Raisman in UCL. They were isolated from olfactory bulb in adult Sprague Dawley rats. OECs were double immunostained for p75NTR (a commonly used maker for OECs as well) and PSA (mAb735), and it was found PSA was widely expressed on OECs (Fig. 6-8). An experiment will be carried out to study whether removal of PSA from OECs by Endo-N treatment will change their interaction with astrocytes.

Fig. 6-8 Expression of PSA on olfactory ensheathing cells.
PSA was detected on OECs with a monoclonal IgG anti-PSA antibody (mAb735). Cells were also immunostained for p75NTR (a marker for OECs as well as for SCs). Scale bar = 25 µm.
6.4 Discussion

Injuries to CNS cause activation of astrocytes, demonstrated by hypertrophy, proliferation, and increased expression of GFAP and CSPGs (Sofroniew and Vinters 2010). Astrocytes also display stress response when they come in contact with SCs both in vitro (Lakatos et al., 2000; Fairless et al., 2005) and in vivo (Lakatos et al., 2003a). Such response of astrocytes will isolate the SC graft and prevent the migration of the transplanted SCs. On the other hand, OECs, although share many biological and morphological characteristics with SCs, were reported not to cause significant stress response to astrocytes, migrate easily inside spinal cord, and intermingle well with the host spinal cells after transplantation (Lakatos et al., 2000; Lakatos et al., 2003a). Such properties of OECs make them a favourable candidate for neural repair (Fairless and Barnett, 2005; Franssen et al., 2007; Raisman et al., 2010). However, the inefficient in vitro expansion of OECs has limited their clinical usage although clinical trials using OECs have been carried out (Mackay-Sim et al., 2008). In this study, we used several in vitro experiments to investigate the interaction between SCs and astrocytes. Our approach is similar to those which have already been used to demonstrate poor migration of SCs in the presence of astrocytes and the hypertrophy induced in astrocytes by contact with SCs (Eng and Ghirnikar, 1994; Wilby et al., 1999). Our results showed that PSA over-expressing SCs gained many of the properties of OECs that are beneficial for transplantation. PST/SCs are able to cross into astrocyte territory and intermingle with astrocytes in confrontation assay, cause less proliferation of astrocytes than GFP/SCs in co-culture assay and do not induce significant level of expression of GFAP and CSPGs in the neighbouring areas of the transplants. Taken together, these results indicate SCs induce astrocytic stress and engineered expression of PSA on SCs significantly changes their interaction with host astrocytes and makes astrocytes more tolerable to the peripheral nerve derived glial cells.

The underlying mechanism for the modification of astrocyte stress response to SCs is still unclear. The astrocyte stress response is reported to be mainly mediated by a FGF family member, and heparan sulfate proteoglycans (HSPGs) may be important for mediating the stress response in astrocytes via FGF2 (Santos-Silva et al., 2007). It has been speculated that the presence of PSA induces a conformational change in the NCAM to produce or enhance the HSPG binding site (Storms and Rutishauser, 1998). Thus, the
hypothesis is that PSA may modulate HSPG binding and then indirectly modify FGF signal pathway in astrocytes to alter astrocyte stress response. However, it remains unclear whether PSA actually binds to HSPGs or indirectly modulates a NCAM-HSPG interaction. Other molecules like N-cadherin, integrin, aggrecan, and ephrins are also reported to be involved in the interaction between astrocytes and SCs and may prevent the intermingling of the two types of cells and the migration of transplanted SCs (Fairless et al., 2005; Afshari et al., 2010a; Afshari et al., 2010b). Since the repulsion force of PSA can attenuate not only homophilic NCAM–NCAM binding but also affects heterophilic binding with other cell surface molecules such as L1, laminin, cadherin, and integrin (Acheson et al., 1991; Fujimoto et al., 2001; Johnson et al., 2005), expression of PSA on SCs may interfere with the interactions of multiple molecules and signal pathways that limit their intermingling with astrocytes and migration.

PSA is expressed on one class of OECs (Franceschini and Barnett, 1996). In this current study, we also showed that PSA-ir was widely expressed on OECs by immunostaining. Taken together, we speculate that PSA might be responsible for the common features shared by OEC and PST/SCs. Since it is difficult to obtain enough OECs for transplantation, the beneficial features of PST/SCs will make them feasible for clinical trials.
Conclusion remarks and future work

SCs as a candidate for cell transplantation for neural repair after neurotrauma have many merits over other types of cells. SCs transplanted into the spinal cord have been shown to promote axon sprouting and remyelination, and improve functional recovery in some animal models of spinal cord injury. However, poor migration, integration and survival of transplanted SCs in the CNS are common problems that limit their therapeutic potential. In this PhD project, I have genetically modified SCs by over-expressing PSA to change their biological properties to improve their survival and enhance their migration and integration within host spinal cord.

I found that PSA-expression significantly enhanced the survival of transplanted SCs in comparison with the GFP-expressing SCs both in short-term (7 days) and longer-term (30 days). Immunosuppression improved the long-term survival of transplanted GFP/SCs; however, it did not further enhance the survival of PST/SCs, indicating that PSA may have similar effect as that of ciclosporin on transplanted GFP/SCs. It has been reported that sialic acids have anti-recognition effect, which is explained by their negative charges in combination with a bulky, hydrophilic molecule (Kelm and Schauer, 1997). I speculate that PSA may mask or shield antigenic sites on SCs, owing to its steric hydroptic structure, to protect them from the attack of host immune system. By using immunohistochmistry, we found there was no difference in either neutrophil or macrophage infiltration to the PST/SC and GFP/SC transplants, indicating that PSA-expression on SCs do not change the responses of inflammatory cells to the transplants.

I further explored other potential underlying mechanisms for the improved survival of PST/SCs both in vivo and in vitro. I firstly investigated whether more PST/SCs proliferated in the spinal cord than GFP/SCs. BrdU incorporation assay showed PSA expression did not increase the proliferation of SCs both in vitro and in vivo. Secondly, I examined whether the improved survival of PST/SCs was due to reduced apoptosis. TUNEL assay of transplanted cells showed that the numbers of TUNEL+ SCs decreased over time (from 1 day to 19 days post transplantation) and there was no difference between PST/SCs and GFP/SCs groups, indicating that PSA-expression may not protect SCs from apoptosis in the time-window of TUNEL assay in this study. It was suggested
that PSA expression is inversely correlated with the expression of $p75^{\text{NTR}}$ in newborn neurons. In this study we also found $p75^{\text{NTR}}$ expression level was significantly decreased in PSA-expressing SCs \textit{in vitro} by immunostaining. $p75^{\text{NTR}}$ has been shown to be involved in SCs death (Boyle et al., 2005; Gascon et al., 2007). I postulate PSA may enhance the survival of transplanted SCs via inducing the down-regulation of $p75^{\text{NTR}}$ expression on SCs. Further study needs to be carried out to investigate the expression level of $p75^{\text{NTR}}$ in transplanted PST/SCs and GFP/SCs to find out whether PSA expression can also reduce the expression of $p75^{\text{NTR}}$ \textit{in vivo}.

Though PSA enhanced the survival of transplanted SCs to a certain degree, the cell death was still extensive over time. Then I tested several factors that might have contributed to the death of transplanted cells \textit{in vitro}. I first investigated the toxicity of glutamate and found SCs were insensitive to high concentration of glutamate. Serum and growth factor withdrawal over 26 h induced significant cell death and PSA expression partially rescued SCs from death induced by serum and growth factor withdrawal. The underlying mechanism is unknown.

By chance, I found that high concentration of ATP could induce significant SC death \textit{in vitro}. As large amount of ATP can be released from cells in spinal cord after spinal cord injury or during cell transplantation procedures, I speculate that ATP may contribute to the death of transplanted SCs in the early stage. ATP induced apoptosis is mediated by the activation of P2X7R and I found that SCs in culture and in sciatic nerves expressed high-level of P2X7R. ATP induced SC death could be blocked by the irreversible P2X7R antagonist, oxATP. As I found out that it took over 4 h for SCs to become sensitive to ATP again after the removal of oxATP, I blocked the P2X7R on SCs and transplanted them into spinal cord. I did find that more oxATP-treated SCs survived than those untreated SCs. One crucial experiment I have started but not finished yet is the transplantation of the SCs from the P2X7R knockout mice to see whether they'll survive better than the SCs from the wild-type mice of the same genetic background. Since P2X7Rs are also expressed on NPCs/NSCs, and β islet cells, enhancing cell survival by blocking P2X7Rs should have a much wider impact in the field of cell therapy. As NPCs/NSCs have been studied as candidates for neural repair and the early cell death is also significant, it would be interesting to find out whether blocking P2X7R can enhance
their survival after transplantation. As oxATP is mildly toxic to cells at the concentration
we used and blockade will be reduced after new P2X7Rs are synthesized, it is imperative
to find new approaches to block the P2X7Rs. One such method is to use LV delivered
short hairpin RNA (shRNA) to knock down P2X7Rs in SCs. Efficient knockdown of
P2X7R in cell lines has been reported in several studies and the sequences can be used
for the design of shRNA. Viral vector mediated knockdown should be stable and
long-lasting. I do not expect long-term knockdown of P2X7Rs will affect their ability for
myelinating regenerating axons as this receptor type is mainly involved in inflammation
and the peripheral nerves of P2X7R knockout mice appear normal. Interestingly, I also
found PSA could partially rescue ATP induced SC death \textit{in vitro} and I also have no
explanation for this phenomenon.

Over-expression of PSA on SCs did not have obvious effect on their migration
ability when they were transplanted into uninjured spinal cord. It is not surprising as SCs
need molecular cues for their migration. Therefore, even PST/SCs are more motile, they
may not migrate without the induction of chemotactic factors. A spinal cord lesion
produced such molecular cues for the transplanted SCs and PST/SCs moved much faster
towards the lesion site. The enhanced migratory capability of PST/SCs may be explained
by the reduced cell adhesion, but it does not exclude other mechanism. One of them
might be that PSA expression on SCs modifies the response of SCs to the chemotactic
factors and initiates stronger migratory potential. Recent findings indicated that NCAM
polysialylation is necessary for the directional response of neural or glial progenitors to a
variety of growth factors (Glaser et al., 2007). TrkB can bind to NCAM directly and
regulate neurite outgrowth (Cassens et al., 2010). As SCs also express TrkB (Frisen et al.,
1993), I speculate that PSA may modify the interactions of signalling molecules such as
NCAM and TrkB in lesioned spinal cord.

Moreover, over-expression of PSA on spinal cord cells facilitated migration of both
PST/SCs and GFP/SCs. It further confirms that reduced cell adhesion by PSA is
beneficial to the migration of transplanted SCs. PSA expression around spinal cord lesion
site could render the glial scar permissive to facilitate the penetration of migrating SCs
into the lesion cavity as it was observed in this study that more PST/SCs migrated into
the lesion cavity. Expression of PSA on spinal cord cells was also shown to promote the
migration of endogenous SCs into the spinal cord. Therefore, expression of PSA on SCs in combination with expression of PSA on spinal cord cells should be an effective strategy to move the SCs transplanted close to the lesion site into the lesion cavity. It has been known that transplantation of SCs directly into the lesion cavity causes significant cell death due to lack of extracellular matrix support and the lack of growth factors. It would be interesting to compare whether more PST/SCs will survive if they are transplanted adjacent to the lesion site instead of directly into the lesion site, which would be more practical clinically.

Using confrontation assay I mimicked the interactions between astrocytes and SCs *in vivo*. Expression of PSA on SCs significantly changed their behaviour when they encountered astrocytes. They were able to cross into astrocytic territory and did not cause hypertrophy of astrocytes. Such dramatic change of their biological property makes them more OEC-like. As it is known that OECs express PSA, it would be interesting to see how OECs will interact with astrocytes in the confrontation assay after the removal of PSA on OECs with Endo-N. The mechanism that PSA regulates the interaction between SCs and astrocytes still remains unclear. Based on the data from current *in vitro* studies, this effect is most likely mediated via direct cell-cell contact. PSA may interfere with the signalling pathways that trigger stress response of astrocytes. The astrocyte stress response is reported to be mainly mediated by a FGF family member (Santos-Silva et al., 2007), and we may examine the activation of its intracellular downstream signalling pathways, such ERK pathway, in astrocytes after confrontation with SCs to see whether PSA can reduce the activation of the pathways.

Last but not the least, the regulation of myelination by PSA over-expression is another issue needs to pay attention to. Due to its bulk volume, it is logical to believe that PSA expression should be in an inverted relationship with myelin formation. (Charles et al., 2000; Jakovcevski et al., 2007). In this study, we found that PSA-ir decreased over time after transplantation, which is in agreement with the results from other studies (Papastefanaki et al., 2007; Bachelin et al., 2010); PST/SCs could still myelinate axons nine weeks after transplantation when the PSA-ir can hardly be detected. PSA down-regulation, observed at later time points, may be a prerequisite for SC differentiation into myelin forming cells. Although the mechanism regulating PSA
expression remains obscure, it might result from post-transcriptional regulation triggered by SC differentiation. As a consequence, over-expression of PSA in SC did not impair their myelinating potential.

In conclusion, the findings from present study demonstrate that PSA expression on SCs is able to enhance their survival after transplantation, facilitate their migration towards lesion site in spinal cord, and improve their integration with astrocytes. Such modification would make SC transplantation more feasible for clinical application in the treatment of neurotrauma.
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