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Extensive cell migration, axon regeneration and improved function with polysialic acid-modified Schwann cells after spinal cord injury

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Abstract

Schwann cells (SC) implantation after spinal cord injury (SCI) promotes axonal regeneration, remyelination repair and functional recovery. Reporative efficacy, however, may be limited due to the inability of SCs to migrate outward from the lesion-implant site. Altering SC cell surface properties by over-expressing polysialic acid (PSA) has been shown to promote SC migration. In the current study, a SCI contusion was used to evaluate the migration, supraspinal axon growth support and functional recovery associated with polysialyltransferase (PST)-over-expressing SCs (PST-GFP SCs) or controls (GFP SCs). Compared to GFP SCs, which remained confined to the injection site at the injury center, PST-GFP SCs migrated across the lesion: host cord interface for distances of up to 4.4 mm within adjacent host tissue. In addition, with PST-GFP SCs, there was extensive serotonergic and corticospinal axon in-growth within the implants that was limited in the GFP SC controls. The enhanced migration of PST-GFP SCs was accompanied by significant growth of these axons caudal to lesion. Animals receiving PST-GFP SCs exhibited improved functional outcome, both in the open-field and on the gridwalk test, over modest improvements provided by GFP SC controls. The current study for the first time demonstrates that a lack of migration by SC may hinder their reparative benefits and that cell surface overexpression of PSA enhances the ability of implanted SCs to associate with and support the growth of corticospinal axons. These results provide further promise that PSA modified SCs will be a potent reparative approach for SCI.

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Keywords

cell transplantation; migration; axon regeneration; scar; functional recovery

Introduction

Numerous experimental studies have reported on the effectiveness of cell therapeutics as a reparative approach for anatomical restitution and locomotor recovery after spinal cord injury (SCI) (Louro and Pearse, 2008; Pearse and Barakat, 2006; Pearse and Bunge, 2006). Schwann cells (SCs), the myelinating glia of the peripheral nervous system, are a cell therapy that has proved to be widely beneficial after experimental SCI (Bunge and Pearse, 2003), reducing tissue damage (Takami et al., 2002) and neuronal cell loss (Schaal et al., 2007), promoting axon re-growth (Pearse et al., 2004a&b; 2007; Xu et al., 1997) and remyelination repair (Kocsis and Waxman, 2007) as well as enhancing functional outcome (Pearse et al., 2004a; Takami et al., 2002). Although SC implants are beneficial in models of experimental SCI, with clear prospects for clinical use (<http://miamiproject.miami.edu>), limitations still constrain their effectiveness, primarily low implanted cell survival (Patel et al., 2010; Pearse et al., 2007) and restricted central nervous system (CNS) migration (Afshari et al., 2010a; Blakemore and Franklin, 2000; Pearse et al., 2007).

Like in the dorsal root entry zone (DREZ), the glial scar that forms around a SCI is a potent restrictive barrier to the outward migration of implanted SCs (Afshari et al., 2010a; Blakemore and Franklin, 2000). The prevention of SC migration from the lesion-implant site may (1) limit their ability to reach axons, particularly supraspinal axons that have retracted after axotomy, and guide them across the injured cord, (2) restrict them from guiding axons to re-enter the caudal spinal cord (Xu et al., 1997) and (3) retard their ability to reach areas of distal demyelination and mediate remyelination repair. Mechanisms that have been implicated in glial scar-mediated restriction of SC migration include extracellular matrix molecules such as the chondroitin sulfate proteoglycan, aggrecan (Afshari et al., 2010a), and diffusible factors including ephrins (Afshari et al., 2010b) or cell adhesion molecules such as N-cadherin (Fairless et al., 2005). To overcome these negative influences and permit SC migration, various strategies have been employed including RNAi knockdown of aggrecan (Afshari et al., 2010a) or N-cadherin (Fairless et al., 2005), EphA receptor block on SCs with EphA4-Fc (Afshari et al., 2010b) as well as the supplementation of either fibronectin (Milner et al., 1997) or neurotrophins (such as NT3) (Yamauchi et al., 2004).

To date the most successful strategy to enhance SC migration over astrocytes in culture and within the injured CNS or peripheral nervous system (PNS) has been to alter their cell-surface with polysialylated neural cell adhesion molecule (PSA-NCAM) (Lavdas et al., 2006; Lou et al., 2011; Papastefanaki et al., 2007). PSA is a large and highly negatively charged glycan which is cross-linked to NCAM by one of two related polysialyltransferase (PST) enzymes: ST8SiaIV (PST) or ST8SiaII (STX) (Eckhardt et al., 1995).

PSA has been shown to play a crucial role in stem cell migration and axon pathfinding during development as evidenced from studies using a PSA-specific endoneuraminidase (Rutishauser, 2008) or PST/STX knockout mice (Angata and Fukuda, 2003). In adult animal

models, we and others have shown that the introduction of PST using viral vectors to the injured CNS can induce PSA and promote repair by allowing axons to re-grow across lesioned tissue (El Maarouf et al., 2006; Lou et al., 2011). Thus, in addition to facilitating SC migration over astrocytes in culture (Luo et al., 2011; Lavdas et al., 2006; Papastefanaki et al., 2007) and *in vivo* (Luo et al., 2011), PSA induction can enhance SC's ability to support axon growth and functional recovery (Papastefanaki et al., 2007).

In the current study, we extend the PST/SC approach in three critical areas: 1) using for the first time the PST expressing, adult-derived SCs in the most commonly-employed and clinically-relevant experimental SCI model, a spinal cord contusion; 2) examining whether PSA modification of implanted SCs allow them to support the growth of corticospinal tract axons, an important descending axonal system for locomotor function in man and an axonal type that SC implants are unable to support the growth of after SCI (Barakat et al., 2005; Pearse et al., 2007); and 3) evaluating the role of PST-expressing SC in SCI by assessing whether the PSA-enhanced SC migration correlates with the observed improvements in axon growth and/or functional recovery.

Materials and Methods

Cell Cultures and Lentiviral Vectors

Schwann cell preparation—Purified populations of SCs were collected from dissociated sciatic nerves of adult female Fischer rats, according to the procedure of Morrissey et al. (1991) with modifications as described by Meijs et al. (2004). SCs were plated on poly-lysine-coated dishes with D10 mitogen media (D10+3M; DMEM+10% FBS, Pen-Strep, 2 μ M forskolin, 20 μ g/ml pituitary extract and 10 ng/ml heregulin). At passage 1, fibroblasts were removed from SC cultures using immunopanning with the Thy 1.1 antibody (ATCC, Manassas, VA). SCs were passaged two more times in D10+3M prior and cryopreserved as stocks for experimental use. Prior to spinal cord implantation, SCs were thawed, grown to 80% confluency and employed at Passage 4. Following this protocol, SC purity was >95% at time of implantation as determined by S100 immunoreactivity.

Construction and introduction of lentiviral vectors into SCs—The cDNA encoding enhanced green fluorescent protein (EGFP) or mouse polysialyltransferase, ST8Sia IV (1,394 bp, Accession NM_001159745), fused to yellow fluorescent protein (YFP-PST) was inserted into a pCS-CG transfer plasmid, between its unique NheI and XhoI cutting sites. Vector preparation was performed as previously described by Follenzi et al. (2000). Briefly, the genes encoding EGFP or PST-YFP were separately sub-cloned into a lentiviral vector (LV) plasmid. This plasmid contained the cytomegalovirus (CMV) promoter to drive transgene expression and the Woodchuck posttranscriptional regulatory element (WPRE) to enhance mRNA transport (Golden et al., 2007). Transfection of plasmids and viral harvesting was conducted in cultured 293T cells. Virus was concentrated by ultracentrifugation at 20,000g and resuspended in phosphate buffered saline (PBS). After this, the viral vectors were titrated for transducing units either on 293T cells or by using an enzyme linked immunosorbent assay (ELISA; Perkin Elmer, Wellesley, MA) for quantifying p24 core protein concentrations, according to manufacturer's instructions. For

these studies the titer of the LV-GFP stock was 7.0×10^7 and the PST-YFP stock was 1.70×10^7 infectious particles. Purified viral vector stocks were stored at -80°C until SC infection.

For *in vitro* and *ex-vivo* infection of SCs with lentiviral vectors (LVs) encoding EGFP and/or a PST-YFP fusion protein, passage 1 SCs at 60–70% confluence were used. SCs were first infected with either LV-GFP or LV-PST-YFP at a multiplicity of infection (MOI) of 50 overnight; media was refreshed the following day and cultures maintained until Passage 3. Preliminary studies using a MOI titration from 20–200 showed that a MOI of 50 for LV-GFP and LV-PST-YFP resulted in an absence of toxicity with a >95% infection of SCs as visualized by EGFP or YFP fluorescence at 48 or 96 hours post-infection, respectively. For PST-YFP SCs that were used for spinal cord implantation, at passage 2, they were co-infected with LV-GFP according to the aforementioned protocol. These cells will be henceforth identified by the nomenclature, PST-GFP SCs. At passage 3, LV infected and expanded SCs were cryopreserved as stocks until experimental use. For *in vitro* and *in vivo* experiments, LV SCs were thawed, grown to 80% confluency and employed at Passage 4.

Western blotting—Following 2X washes in HBSS, plates of SCs were incubated for 10 min at 40°C in 500 μl of lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $\text{Na}_2\text{-EDTA}$, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ leupeptin and 1 mM PMSF] and cells removed using a cell scraper. To demonstrate PSA antibody specificity, some SC plates were first treated with 1U endoneuraminidase-N (EndoN) at 37°C for 1 h prior to lysing to remove PSA from NCAM. Collected SC lysates were centrifuged for 10 min ($14,000 \times g$) at 40°C and the protein concentration of supernatants quantified using the Bradford Assay (Bio-Rad Laboratories, CA). Aliquots containing 30 μg protein were loaded for one-dimensional 7% SDS-polyacrylamide gel electrophoresis (Pearse et al., 2001) and proteins transferred to PVDF membranes at 25V overnight at 4°C . Membranes were blocked in 1X TBST containing 3% BSA, then incubated in primary mouse monoclonal anti-PSA-NCAM antibody (1:1000; Chemicon Inc., Pittsburgh, PA), followed by HRP-conjugated goat anti-mouse IgG (1:5000; Jackson ImmunoResearch Laboratories Inc., PA). Membranes were then incubated with reagents from the SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL) for 3 min and exposed to Kodak X-OMAT films (Eastman Kodak Co., Rochester, NY). Quantification of PSA-NCAM signal was performed in a Fluor-S Multi Imager (Bio-Rad). Band densitometry measurements were made using Quantity One software version 4.2.0 (Bio-Rad). To ensure consistent loading of equal protein amounts, blots were stripped and then incubated with β -actin mouse monoclonal antibody (1:10,000, Sigma). β -actin signal was quantified as described above.

Immunocytochemistry—GFP or PST-GFP SCs were grown to 80% confluency in 4-well chamber slides. SCs were washed with 2X HBSS, fixed with 4% paraformaldehyde and maintained in PBS prior to staining. Fixed SCs were immunostained according to previous methods (Pearse et al., 2004c) using the primary mouse monoclonal anti-PSA-NCAM antibody (1:200, Chemicon) and goat anti-mouse Alexa-594-conjugated IgG (1:200,

Invitrogen, Carlsbad, CA) for visualization under fluorescent microscopy. Cell nuclei were stained with Hoechst (Sigma-Aldrich, St. Louis, MO).

Animals

Adult female Fischer rats (Harlan Co, n = 34; 180–200 g) were housed according to NIH and The Guide for the Care and Use of Animals. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. Prior to surgery, rats were anesthetized (45 mg/kg ketamine, 5 mg/kg xylazine) by intraperitoneal injection.

Moderate thoracic contusion injury—Contusion injury was induced by the MASCIS weight drop device developed at New York University (Gruner, 1992). A laminectomy performed at thoracic vertebra T8 exposed the dorsal surface of the spinal cord underneath (T9) without disrupting the dura mater. The exposed spinal cord was moderately injured by dropping a 10.0 g rod from a height of 25.0 mm. The contusion impact height, velocity and compression were monitored. Animals (n=4) were excluded immediately when height or velocity errors exceeded 7% or if the compression distance was not within the range of 1.75 to 2.25 mm. After injury, the muscles were sutured in layers and the skin closed with metal wound clips. The rats were allowed to recover in a warmed cage with water and food easily accessible. Post-operative care, including the administration of antibiotics, analgesics and fluids, was performed as described elsewhere (Pearse et al., 2007). At 1 week post-injury, rats were randomly assigned into one of 3 treatment groups; (i) injured, no implantation (n=10); (ii) injured, GFP SC implantation (n=10) or; (iii) injured, PST-GFP SC implantation (n=10). One animal each from groups (ii) and (iii) died during the implantation surgery. One animal from group (i) died during tracing. Therefore an n of 9 animals per group survived to endpoint and were used for behavioral and histological analyses.

Intraspinal implantation of SCs—Prior to implantation, LV GFP or LV PST-GFP infected SCs were harvested from culture via trypsinization, centrifuged, resuspended, and counted. After counting, SCs were prepared as aliquots at a density of 3.3×10^5 cells/ μ l in DMEM-F12 medium and were kept on ice (<2 hours) prior to implantation. One week after SCI animals were re-anesthetized and the original site of laminectomy re-exposed for the injection of 2×10^6 SCs in DMEM media (6 μ l) into the injury epicenter. Injections were performed using a 10 μ l siliconized Hamilton syringe with a pulled, beveled glass pipette tip (100 μ m diameter), held in a micromanipulator with a micro-injector at a rate of 2 μ l/min (World Precision Instruments Ltd, Sarasota, FL). The injection pipette was kept in place for an additional 3 min to minimize leakage upon withdrawal. After the injections, the muscle layers and the skin were closed separately and animals received post-operative care as described above. Injury-only controls received no spinal cord injections.

Anterograde tract tracing—For anterograde tracing, 9 weeks post-injury (8 weeks post-implantation), following endpoint behavioral testing, rats were re-anesthetized and 10% BDA (Molecular Probes, Eugene, OR) was stereotactically injected at 4 sites (Bregma –1.5, –2, –2.5, –3 mm, 2.5 mm lateral to the midline, depth 1 mm, 0.5 μ l each) bilaterally into the hind limb area of the motor cortex. Injections were made using a 5 μ l Hamilton syringe with a pulled, beveled glass pipette tip (20 μ m diameter), held in a micromanipulator with a

micro-injector at a rate of 0.25 μ l/min (World Precision Instruments Ltd, Sarasota, FL). The injection pipette was kept in place for an additional 1 min to minimize leakage upon withdrawal of the syringe and gelfoam was used to cover the borehole upon completion of the injections. Brains from anterogradely-traced animals were sectioned and examined to ensure accurate tracer injections into the hind limb area of the motor cortex and adequate tracer diffusion in each animal. No animals were excluded based upon these criteria prior to analyses.

Histology

At 11 weeks after injury, rats were deeply anesthetized (70 mg/kg ketamine, 10 mg/kg xylazine), 300 U heparin was injected into the spleen and the animals transcardially perfused first with 300 ml of cold saline and then 500 ml of phosphate-buffered 4% paraformaldehyde (0.1 M, pH 7.4; Lo et al., 2009). The T7–T11 thoracic spinal cord (20-mm-long piece) that contained the complete lesion and SC implant was dissected, postfixed in paraformaldehyde overnight and placed in 30% w/v sucrose for 48 h for cryoprotection during histological processing. Spinal cords were sectioned sagittally on a cryostat into 5 series at 20 μ m (each section was separated by a 100- μ m interval) for immunochemistry and stereological quantification of host responses, SC migration and axon growth. The cerebral cortex was also dissected post-fixation and 40- μ m-thick transverse sections were prepared with a freezing microtome and every tenth transverse section was stained and mounted for analysis of anterograde tracer injection sites.

Immunohistochemistry—Every other fifth sagittal section (200 μ m interval between sections) was immunochemically stained as previously described (Barakat et al., 2005). The following primary antibodies were used; monoclonal antibody anti-gial fibrillary acidic protein (GFAP, SMI-22, 1:1,000; Sternberger Monoclonals, Lutherville, MA) or the polyclonal antibodies: anti-GFP (Rabbit polyclonal, 1:200; Santa Cruz, CA), anti-serotonin (5-HT, 1:5,000; Immunostar, Hudson, WI), anti-Neurofilament-200 (Rabbit polyclonal, 1:500; Encor Biotechnology Inc., Gainesville, Florida), anti-CSPG (mouse monoclonal, 1:200; Sigma), anti-S100 (mouse monoclonal, 1:200; Sigma) or AlexaFluor-594 conjugated streptavidin (1:200 dilution, Invitrogen; to identify BDA-traced CST axons). Fluorescent conjugated secondary antibodies (goat anti-rabbit AlexaFluor-488 or 594 or goat anti-mouse 594; 1:200; Molecular Probes) were used for signal visualization by fluorescent microscopy. Slides were coverslipped with Vectashield medium (Vector Labs, Burlingame, CA).

Image Analysis

For image analysis, stained sections were viewed with Olympus Fluorescence microscope (Olympus, Fluoview FV 1000) or a confocal laser-scanning microscope (Carl Zeiss, LSM 500). All profile counts or immunoreactivity measurements were conducted by individuals who were blinded to the experimental procedures performed on each animal. Images were taken with an Olympus BX51 microscope and Meander scan software (MicroBrightfield, Williston, VT). For presented images, the tonal range and sharpness (smart sharpen, 0.9 pixels) of the Tiff files were normalized using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA).

Quantification of implanted SC numbers within the injured spinal cord—In one series of mounted sagittal sections, GFP or PST-GFP SCs, visualized by fluorescent signal, were counted using an unbiased method employing computer-assisted microscopy and StereoInvestigator software (MicroBrightField) throughout the 3D structure of the spinal cord as performed previously (Pearse et al., 2007). Briefly, in each section the boundary of the SC implant was first traced with a 20X objective and logged into the StereoInvestigator software using serial section manager, which tracked the positions of each section (~10–12 sections per spinal cord at 200 μm intervals) within the Z-axis of the 3D reconstruction of the spinal cord. Then, using optical fractionator, the traced contour of each section was divided into $50 \times 50 \mu\text{m}^2$ counting frames. Next, the grid size was automatically determined by the software to permit counting of 35 sampling sites within each traced contour of each section. In each sampling site the number of GFP-positive cells with Hoechst (fluorescence) stained nuclei were then marked with a dissector probe under a 60X objective. Algorithms for estimating the Z-stack total counts of positive cells within the 3D structure of each spinal cord were performed according to StereoInvestigator 3.0 (MicroBrightfield). The coefficient of error (CE) (Gundersen) for each estimated count was less than 10%.

Evaluation of SC migration beyond the lesion: host cord interface—To assess the maximal extent of *in vivo* migration of GFP or PST-GFP SCs following transplantation into the injured cord, sections that contained GFP-labeled cells were immunostained for GFAP to define the borders of the injury site. Using computer assisted microscopy and stereological software (MicroBrightfield) the distance of the leading front of GFP or PST-GFP SCs (defined as being a group of at least 10 cells at equivalent distance) was measured in each section from the caudal and rostral lesion: host cord GFAP-positive borders. The maximal caudal and rostral migration distances measured across the sections analyzed was recorded as the maximal migration for that animal. Additionally, maximal caudal and rostral migration distances were added and averaged to provide a total migration distance value as some animals exhibited more rostral cell migration and others more caudal cell migration.

Measurement of serotonergic and corticospinal tract axon growth within and beyond the SC implant—BDA-labeled or 5-HT-immunostained axons were examined in every fifth section of the T8–10 cord segments, i.e. every 200 μm interval spanning the width of the spinal cord at 10 weeks post-transplantation. Labeled axons were visualized using fluorescence microscopy. The number of BDA- or 5-HT-positive axons was determined under oil immersion with a 60X objective by counting all labeled axon profiles within a 5 μm thick strip perpendicular to the center of the GFP-positive SC implant and at distances of 500, 1000 and 1500 μm from the caudal lesion: host cord interface. The lesion: host cord interface was demarcated by GFAP immunostaining and all axons through the Z-plane thickness of the section (~20 μm) within the strip were counted. For each region in each section the counts of axon profiles were expressed per mm^2 and then averaged across sections to provide an axon count for each animal. Tracing efficiency was also examined across animals by examining dye intensity in the dorsal columns of five transverse sections from the C2 spinal cord from each animal. Images of the dorsal columns in each of the sections were analyzed using Metamorph (Molecular Devices). The software was used to measure the total pixel intensity of the dye staining in each image in arbitrary units. These

values were then averaged over the five sections to give a mean BDA intensity for each animal as a measure of tracing efficiency. No animals were found outside of 1 standard deviation of the mean, a predictive indicator of poor tracing efficiency.

Chondroitin sulfate proteoglycan production and astrogliosis—Chondroitin sulfate proteoglycan (CSPG) deposition, identified by CS-56 immunoreactivity, and astrogliosis, determined by GFAP staining, in host cord areas within 500 μ m and 2 mm caudal to the SC implants, were measured using Metamorph Imaging software (Molecular Devices), according to previously described methods (Pearse et al., 2004b). In brief, three sections from the center of the SC implant from each animal were imaged on an Olympus BX51 microscope (Olympus America Inc.), the region of positive staining was specifically demarcated after conversion to black and white and then the average per pixel intensity within the stained area was obtained as arbitrary units using Metamorph (Molecular Devices). These values were averaged among the three sections to provide a value of immunoreactive intensity each for CS56 and GFAP for each region in each animal.

Behavioral testing

Prior to injury, all animals were evaluated to establish baseline scores on the behavioral tests employed. After injury, animals received temporal testing for each functional measure as outlined below. All behavioral scoring was carried out by two observers who were blinded to the experimental procedures performed on each animal.

BBB open field locomotor score—Hind limb performance was evaluated with the open field locomotor test developed by Basso et al. (1996). Two observers, unaware of the experimental procedures, performed the test weekly for 9 weeks. A BBB sub-score was also used as previously described (Pearse et al., 2007).

Grid walk test—Deficits in descending motor control were examined pre-injury and at 2, 4, 6 and 8 weeks post-SC implantation by assessing navigation across a 1-m-long runway with irregularly assigned gaps (0.5–5 cm) between round metal bars, as described previously (Pearse et al., 2007).

Statistical analysis

Statistical analysis was performed using Graphpad Version 4.0 (Graphpad Software, Inc., La Jolla, CA). A repeated-measures ANOVA with a Bonferroni posttest was used for comparisons of weekly BBB and BBB sub-scores as well as gridwalk errors, SC migration and caudal corticospinal tract axon growth between treatment and control groups. All other data set comparisons between SC groups were subjected to a Student's t-test. Differences were accepted as statistically significant at * $p < 0.05$, ** $p < 0.01$ or and *** $p < 0.001$. All errors are given as SEM. For independent linear correlation analyses between individual tests of functional outcome and SC migration or caudal axon growth by expression of Pearson correlation coefficients (R), standard R tables were employed for calculation of statistical significance from degrees of freedom ($n - 2$) as performed previously (Pearse et al., 2005).

Results

Infection of SCs with PST-YFP alters cell morphology and significantly increases surface PSA

Following infection SCs with LV PST-YFP, inherent fluorescence of the fusion protein was detected at high levels in the Golgi and cytoplasm by 96 hours (Fig. 1A). The transduced SCs continued to express PST-YFP for at least 10 passages in culture. Unlike GFP SCs, which exhibited a spindle morphology and formed a swirling pattern with parallel cell alignment when grown to confluence on poly-lysine (Fig. 1B), PST-YFP SCs, had a multi-polar shape and formed a mesh-like cellular network (Fig. 1C). Compared to GFP SCs, which did not show punctate PSA immunoreactivity upon their cell surface (Fig. 1D), PST-YFP SCs showed a robust surface expression of PSA-NCAM (Fig. 1E). The use of endoneuraminidase-N (EndoN), which cleaves PSA from NCAM, demonstrated the specificity of PSA immunoreactivity (Fig. 1F).

The ability of LV PST-YFP infection to enhance the production of PSA by adult SCs was further confirmed by immunoblotting (Fig. 2). Low levels of PSA were observed in LV GFP SCs while LV PST-YFP infection produced a 265% increase in PSA levels ($p < 0.001$; $n = 3$); pre-treatment with EndoN prior to cell lysis removed PSA, confirming the specificity of the band detected at a molecular weight of 150kDa.

No significant change in implanted SC numbers is seen with PST expression

Previous work has demonstrated that approximately 15–20% of labeled, implanted SCs survive when grafted sub-acutely following moderate contusive SCI in a Fischer rat syngeneic paradigm (Hill et al., 2007; Pearse et al., 2007). In animals receiving GFP SCs, an average number of $392,400 \pm 87,700$ SCs was counted (19.6% survival), while for PST-GFP SCs, $433,500 \pm 93,200$ SCs were detected (21.7% survival) within the injured spinal cord (Fig. 3). No statistically significant difference in cell survival/persistence within the injured spinal cord environment was therefore found when implanted SCs had PSA upon their surface.

Greater SC migration into the adjacent host spinal cord and intermingling with host astrocytes are observed when they express PST

Implanted SCs are largely restricted in their migration from the lesion site into adjacent host cord tissue unless they gain access to the central canal or subdural space during the injection procedure (Fig. 4; Pearse et al., 2007). In animals receiving GFP SCs, implanted cells were found predominantly within the lesion site (Fig. 4A) though in some cases significant rostral dispersion was observed when the SCs had gained access to the central canal. GFP SCs were not observed intermingling with astrocytes except within the immediate penumbra of the lesion. In contrast, animals receiving PST-GFP SCs exhibited significant rostral and caudal migration (up to 4.4 mm distal from the GFAP:lesion border), within the surrounding host cord tissue (Fig. 4B, D), and the intermingling of PST-GFP SCs with host astrocytes was observed many millimeters from the lesion site (Fig. 4D). Extensive migration of PST-GFP SCs into the adjacent host spinal cord left the injury epicenter of some animals with few labeled, implanted SCs, though the lesion sites remained filled with non-labeled cells.

Immunohistochemistry using the SC marker S100 revealed that the lesion sites of PST-GFP SC animals were densely populated with endogenous SCs that provided a continuum across the injury site between outward migrating PST-GFP SCs (Fig. 4C).

Implantation of PST-GFP SCs does not alter host cord GFAP and CS56 immunoreactivity compared to GFP SC controls

Images of CS56 staining 2 mm caudal to the lesion interface of GFP SC and PST-GFP SC implanted animals are shown in Fig. 5A–B. Metamorph analysis of three medial sections per animal, averaged across the animals from each cell implant cohort, revealed that the average per pixel GFAP immunoreactive intensity at the caudal interface after GFP SC implantation was 164.6 ± 6.1 arbitrary units (a.i.; Fig. 5C). No significant difference in GFAP immunoreactive intensity was observed between GFP SCs and PST-GFP SCs, 159.6 ± 6.0 a.i. ($p > 0.05$; Fig. 5). Similarly, no difference in CS56 immunoreactive intensity at the caudal interface was observed among GFP SCs (59.1 ± 4.2 a.i.) and PST-GFP SCs (63.0 ± 4.1 a.i.; $p > 0.05$) implanted spinal cords (Fig. 5C). Although no significance difference in either GFAP or CS56 immunoreactive intensity was observed among the different cell cohorts at 2 mm caudal to the lesion: host interface, near the leading edge of the PST-GFP SC migratory front, there was a trend for less CS56 immunoreactivity in the PST-GFP SC implanted animals (Fig. 5C. GFP-SCs, 14.4 ± 1.2 a.i.; PST-GFP SCs, 9.8 ± 2.1 a.i.; $p = 0.059$).

PST-GFP SC implants support extensive serotonergic and corticospinal tract axon in-growth and out-growth

We have previously reported the poor growth response of supraspinal axon populations, such as serotonergic (5-HT) and corticospinal tract (CST) axons, following thoracic SCI and SC implantation (Pearse et al., 2004a&b; 2007). Quantification of immunostained serotonergic axons within GFP SC implants showed limited numbers of axons, averaging 20.9 ± 7.8 5-HT axons/mm² (Fig. 6A, D). Significantly more growth was observed within PST-GFP SCs implants, with 431.2 ± 112.0 5-HT axons/mm² (20.6-fold enhancement; $p < 0.01$) (Fig. 6B, D). Numerous 5-HT axons were seen extending largely in the extracellular spaces surrounding PST-GFP SCs, though occasionally PST-GFP SCs appeared to be in close association with 5-HT axons (Fig. 6C; white arrows). In addition, more spared or re-growing 5-HT axons were observed >1000 μ m distal from the caudal lesion: host interface in PST-GFP implanted animals (704.9 ± 423.5 5-HT axons/mm², $p < 0.05$) compared to GFP SC controls (155.5 ± 96.7 5-HT axons/mm²; Fig. 6D).

Similarly, while GFP SC implants only provided for the growth of 29.4 ± 13.5 CST axons/mm² (Fig. 7A), significantly greater growth occurred into PST-GFP SC implants, 1107.4 ± 502.7 CST axons/mm² (37.7-fold enhancement; $p < 0.05$; Fig. 7B–E). Confocal microscopic analysis revealed that many CST axons were aligned with PST-GFP SCs within the implant-lesion (Fig. 7C, D; white arrows).

We further quantified CST axon growth/sparing caudal to the SC implants at distances of 500, 1,000 and 1,500 μ m. The numbers of CST axons found caudal to GFP SC implants were 10.4 ± 5.4 (500 μ m), 9.8 ± 5.7 (1,000 μ m) and 8.5 ± 4.2 (1,500 μ m) axons/mm² (Fig. 8B). Following PST-GFP SC implantation, significantly higher numbers of CST axons (>10 -

fold) were found at all evaluated distances caudal to the implants; 125.4 ± 60.1 (500 μm , $p < 0.05$), 139.1 ± 60.7 (1,000 μm , $p < 0.05$) and 101.5 ± 49.7 (1,500 μm , $p < 0.05$) axons/ mm^2 (Fig. 8A–B). The growth of numerous CST axons from the leading caudal edge of the PST-GFP SC implant can be seen in Figure 8A.

Animals receiving PST-GFP SC implants show significant improvements in open-field locomotion and foot placement

Although no significant differences existed between treatment and control groups during the initial 1 to 2 weeks post-injury (Fig. 9A), beginning at 3 or 4 weeks post-injury, respectively, a significant improvement in BBB scores was observed between PST-GFP SCs (4 weeks; 10.5 ± 0.5 , $p < 0.01$) or GFP SCs (4 weeks; 9.4 ± 0.3 , $p < 0.05$) and the SCI only controls (4 weeks; 7.6 ± 0.2 ; Fig. 9A). While for GFP SCs this significant improvement in locomotion over SCI only controls was transient (weeks 4 to 6), persistent enhancement of BBB scores with PST-GFP SCs was seen until endpoint (week 9). At study endpoint, BBB scores for the PST-GFP SC group (12.3 ± 0.6) were significantly greater than both GFP SC (10.8 ± 0.3 , $p < 0.05$) and SCI only controls (10.1 ± 0.2 , $p < 0.01$; Fig. 9A).

The employment of a BBB subscore, as we have described previously (Pearse et al., 2007), to evaluate foot positioning and placement, showed no difference over time post-SCI between the GFP SC implant group and the SCI only controls (0.7 ± 0.2 versus 0.4 ± 0.2 , $p > 0.05$; at endpoint; Fig. 9B). In contrast, PST-GFP SCs exhibited significant improvement in BBB subscores beginning at 7 weeks post-SCI versus injury only controls and continuing through endpoint (4.0 ± 1.3 , $p < 0.05$; Fig. 9B). PST-GFP SCs also showed significant improvement in BBB subscores over GFP SC controls at endpoint ($p < 0.05$).

Lastly, the horizontal, irregularly-spaced gridwalk test was used to evaluate differences in foot placement on a challenging behavioral task. While a reduction in footfall errors through endpoint was not observed with GFP SCs (6.5 ± 0.6 errors) versus SCI only controls through endpoint (7.4 ± 0.5 errors), an improvement was observed with PST-GFP SCs from week 4 that persisted to endpoint (3.8 ± 0.7 errors, $p < 0.01$ versus SCI only; $p < 0.05$ versus GFP SCs, at week 8 post-implantation; Fig. 9C).

Linear correlation analysis shows significant correlation between SC caudal migration or caudal CST axon growth and improved function on the gridwalk test

To identify whether a relationship existed between anatomical changes and the observed functional changes, correlative analysis was performed. Endpoint data from animals subjected to contusive SCI and SC implantation (both GFP SC and PST-GFP SC cohorts) was used (Table 1; Fig. 10). No significant correlation was observed between caudal CST axon presence, caudal 5-HT axon presence or SC migration and open-field locomotor scores (BBB scores and subscores; $R < 0.468$; Table 1). Significant correlation was seen, however, between the distance of caudal SC migration and the number of gridwalk footfall errors (inverse; $R = 0.4705$, $p < 0.05$, Fig. 10A) as well as between the amount of caudal CST axon present and the number of gridwalk footfall errors (inverse; $R = 0.5712$, $p < 0.05$, Fig. 10B). Interestingly, no correlation was observed between caudal 5-HT axon presence and the

number of gridwalk footfall errors ($R=0.1673$, $p>0.05$, Table 1). Relationships were demonstrated by evidence of a significant Pearson correlation coefficient.

Discussion

Our findings demonstrate that lentiviral introduction of PST into Schwann cells results in cell surface PSA expression which, following acute implantation of PST SCs into a spinal cord contusion, augments their migration across the inhibitory astroglial environment. This study therefore extends similar results obtained with spinal cord compression and crush injuries (Lou et al., 2011; Papastefanaki et al., 2007), into a more clinically-relevant SCI contusion model. PSA does not have in and of itself the ability to instruct cells to migrate, but rather insulates them from interactions that can restrict their movement, thereby unleashing a migratory capacity instigated probably by cues present within adjacent host spinal cord tissue. The augmented SC emigration from the transplant allows SCs to guide axons from the lesion site as well as increases the chance that axons encounter growth-supporting elements outside the lesion site. In view of the PSA biology during development, in addition to promoting SCs' spatial availability for regenerating axons, this polymer should also enhance their support for axonal growth (Rutishauser, 2008).

In agreement with a previous report (Papastefanaki et al. 2007), which showed an acute benefit of STX-SCs, we found that implantation of PST SCs results in an improvement of gross motor function (hind limb locomotor function as assessed by BBB score analysis); such enhancement persisted through 8 weeks post-implantation. In addition, our studies showed a prominent effect on fine motor skills as measured by the grid walk test. Moreover, the improvement in foot placement on the grid walk test was found to significantly correlate with both the enhancement of SC migration out of the injury-implant site and with the extent of regrowth of corticospinal tract fibers, but not serotonergic axons, into the caudal cord. This study therefore has provided evidence for the role of a SC bridge, produced by enhanced cell migration, to assist the growth of a specific fiber tract (CST) with a precise and positive corresponding functional outcome, improved foot placement on the gridwalk test, a task known to be associated with the CST (Metz and Whishaw, 2002). Further work, however, is needed to identify if these regenerating CST axons form functional synapses on appropriate caudal targets that are responsible for coordinating specific hindlimb movements. It is also likely that plasticity in other axonal systems, both local and descending, produced by PST SC implants but not investigated in the current study, have also contributed to these functional improvements. In addition to the CST, these include the rubrospinal tract which mediates digit positioning, ascending sensory axons which provide tactile feedback during paw placement, and intraspinal neuronal networks which are important for mediating postural support between the limbs and the trunk during ladder rung walking (Metz and Whishaw, 2002).

It is interesting to note that the correlations observed between both SC migration and caudal CST regrowth and the grid walk test were not observed in comparisons with gross motor BBB tests. Gross motor recovery depends on various sensory-motor tracts and may be more subject to spared axons, collateral sprouting and local spinal circuitry (Bradbury and McMahon, 2006) than the fine motor dexterity required for the grid walk, which more

axons and the ensuing restoration or strengthening of neurotransmission could therefore contribute to the observed improvements in functional outcomes with PST SCs. *In vitro* studies have shown that PSA expression on SCs does not alter their ability to myelinate axons of dorsal root ganglion neurons (Lavdas et al., 2006; Papastefanaki et al., 2007), although other studies have reported that PSA expression negatively correlates with CNS myelination (Charles et al., 2000; Koutsoudaki et al., 2010). Future immuno-electromicroscopic evaluation of labeled PST SC myelination ability *in vivo* and the measurement of electrophysiological changes across the lesion site may enable determination of whether axon remyelination contributes to the functional efficacy of PST SC mediated repair after SCI.

Studies have suggested that PSA can interact with other potentially relevant substances, such as BDNF (Muller et al., 2000), heparin sulfate proteoglycans (Storms and Rutishauser, 1998) and extracellular histone H1 (Mishra et al., 2010), which might extend its effects beyond regulation of contact-dependent cell interactions. In addition, Li et al., (2011) recently demonstrated that PSA-NCAM promoted cell migration via a fibroblast growth factor receptor (FGFR)-dependent mechanism mediated by focal adhesion kinase (FAK) and extracellular signal-regulated kinase 1/2 (ERK 1/2). This cell migration occurred independent of PSA-NCAM's effects on cell adhesion properties. Similarly, it could be hypothesized that our results could be derived by secondary changes in ECM expression within the injury milieu, such as of CSPGs, produced by PSA-alteration of SC cell interactions with their environment. However, we did not observe a significant change in CSPG levels or gliosis with grafting of PST SC, suggesting that the primary role of the PSA stems from a direct effect on the SCs and axons.

Although there was significant correlation between caudal SC migration, CST axon re-growth and improvements in paw placement, it is not clear whether PSA surface production directly enhanced the ability of SCs to support axon growth through altered contact-dependent interactions, and/or if PSA worked indirectly by facilitating SC migration and allowing them to then guide axons into and from the lesion. Our previous work with PSA modification of SCI scar astrocytes (El Maarouf et al., 2006) also showed that PSA could enhance regeneration of CST axons through the scar. Unlike the present work with PSA SCs, the PSA-positive scar astrocytes did not leave the lesion site, and CST regrowth was limited to the vicinity of the lesion. Therefore it remains reasonable to suggest that the remarkable increase in SC migration is at least necessary (but perhaps not sufficient) to explain the robust caudal CST and 5-HT axon regeneration after SCI. In addition to studies that have examined the effects of enhanced PSA expression, other work has investigated *in vivo* axon behavior after PSA removal, specifically in developing tissues that normally expresses a high level of PSA. In the different neural systems studied to date; optic axons in tectal regions (Yin et al., 1995), hippocampal mossy fibers in CA3 (Seki and Rutishauser, 1998) and spinal cord sensory or motor neurons (Franz et al., 2005; Honig and Rutishauser, 1996), PSA removal does not decrease the extent of axon growth, but rather changes the local behavior of growing axons. By contrast, removal of PSA from sites of CNS cell migration, in the subventricular zone (SVZ) and forebrain, results in a dramatic decrease in migration along normal routes (Battista and Rutishauser, 2010). Collectively these studies

highlight the importance of PSA in modifying not only the behavior of the neural component upon which it is expressed but also the responses of the local environment to the PSA modified neural component.

The current study for the first time demonstrates the ability of highly migratory, PSA-modified SCs to support long-term functional improvements not only in gross locomotor function but also in foot placement on a more challenging behavioral task, the horizontal ladder, after a clinically-relevant contusive SCI. Accompanying enhanced functional recovery was a robust regrowth of serotonergic and corticospinal tract axons into and caudal to the implants that was not observed with unmodified SCs and, which for CST axons, is usually very limited even with other cellular and combinatory therapeutic approaches. Importantly we demonstrate that there exists a strong correlation between both caudal PSA SC migration and caudal CST axon growth and the observed functional improvements on the horizontal ladder task. From these findings, it is clear that the modification of SCs to express PSA has significant potential for increasing the therapeutic efficacy of this cellular approach as neurorepair strategy following SCI.

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Figure 1. Expression of PST-YFP in adult-derived SCs by lentiviral vector transfer results in high infection rates and robust surface production of PSA-NCAM

A. Expression of the PST-YFP fusion gene results in fluorescent protein production with localization in the cytoplasm of SCs, in particular within the Golgi apparatus; the infection rate was >80% by 96h as visualized by YFP labeling. **B–C.** Compared to GFP SC controls (**B**), which exhibit a spindle morphology and swirling cell pattern with parallel cell alignment when cultured on polylysine, PST-YFP SCs (**C**) show a multipolar phenotype, larger cell bodies and an absence of alignment. **D–F.** GFP SCs (**D**) exhibit very little surface PSA-NCAM compared to those SCs expressing PST-YFP (**E**). The specificity of PSA-NCAM immunoreactivity on the surface of PST-YFP SCs was confirmed through treatment with endoneuraminidase-N (EndoN; **F**). Scale bar = 50 μ m (A–C) or 30 μ m (D–F).

Figure 2. PST-GFP expressing, adult-derived SCs have high PSA levels compared to GFP SC controls as measured by quantitative western blot

Compared to GFP SC control lysates in which negligible levels of PSA are seen, PST-GFP expressing SC lysates show a prominent PSA band at 150kDa following immunoblotting with a PSA specific antibody, a result confirmed by quantitative densitometry measurements. The specificity of PSA immunoreactivity was confirmed through treatment and removal of PSA with EndoN. All PSA bands were normalized to β-actin. Statistical significance indicated at *** $p < 0.001$.

Figure 3. No significant change in implanted SC numbers are seen with PST expression
Stereological quantification of fluorescently-labeled, implanted SCs within the lesion site and adjacent host spinal cord at 8wk showed no significant difference in SC numbers between PST-GFP SC and GFP SC controls.

Figure 4. PST-GFP SCs exhibit greater migration and adjacent host caudal cord penetration than GFP SC controls

A–B. In comparison to GFP SC controls (**A**), PST-GFP SCs (**B**) readily migrate for significant distances along the rostral-caudal axis. Pictured is the animal that exhibited the greatest PST-GFP SC migration, in which many SCs had migrated from the injury-implant site, though, as shown in (E), the extent of PST-GFP SC migration varied among individual animals. **C.** The lesion epicenter, following outward migration of PST-GFP SCs (**green**) into the adjacent spinal cord, becomes populated with S100+ host SCs (**red**). **D.** Extensive intermingling of PST-YFP SCs and GFAP immunoreactive astrocytes are observed at the leading edge of the migratory front of the implanted SCs, 4 mm from the rostral lesion:GFAP host cord interface. **E.** Measurement of the distance between the lesion: GFAP host cord interface and the leading migratory front of implanted SCs, rostral or caudal to the injury site, shows that PST-GFP SCs (black circles) have greater migration into adjacent host spinal cord tissue than GFP SC controls (white circles). Scale bars = 500 and 20 μm .

Figure 5. Implantation of PST-GFP SCs does not alter the injury scar milieu compared to GFP SCs as evidenced by no significant changes GFAP and CS56 immunoreactivity

A–B. CS56 immunostaining to identify spatial CSPG localization within comparative regions, 2 mm distal to the caudal edge of the lesion, in GFP SC (**A**) and PST-GFP SC (**B**) implanted animals. Scale bar = 40 μ m. **C.** Measurements of the average immunoreactivity intensity along the caudal lesion: host cord border and a region 2 mm distal to the caudal interface showed no difference in GFAP and CS56 immunoreactivity between animals that received GFP SC implants and those receiving PST-GFP SC implants.

Figure 6. PST-GFP SCs support extensive serotonergic axon in-growth compared to GFP SC implants as well as greater 5-HT axon sparing/growth caudal to the implant
 Immunohistochemical detection of serotonergic axons (red) using 5-HT show that while GFP SC implants within the injured cord support the in-growth of very few axons (A, D), many more are found penetrating the center of PST-GFP SC implants (B, higher magnification of the SC implant in C, D). Significantly more spared or re-growing 5-HT axons are also seen 1,000–1,500 μ m distal to the caudal lesion interface in animals treated with PST-GFP SCs. White arrows identify 5-HT axons in close association with SCs. Scale bar = 40 μ m. Statistical significance indicated at * $p < 0.05$.

Immunohistochemical detection CST axons (**red**) show that while GFP SC implants within the injured cord support the in-growth of very few CST axons (**A, E**), many more CST axons are found penetrating the center of PST-GFP SC implants (**B** and higher magnification of the center of the SC implant in **C** and **D** [ortho view], **E**). White arrows indentify labeled CST axons in close association with SCs. Scale bar = 50 μ m. Statistical significance indicated at * p <0.05.

Figure 8. The growth of numerous corticospinal tract axons caudal to the injury-implant site are seen in some animals receiving PST-GFP SCs, but not in those injected with GFP SCs

A. In an animal receiving a PST-GFP SC implant, numerous CST axons (red) are seen crossing the caudal edge of the PST-GFP SC (green) migratory front and these CST axons continue to grow into the caudal host cord. Yellow arrows identify CST axons caudal to the SC implant (implant border, dotted white line). Quantification of CST axons caudal to the lesion: host cord interface shows that while GFP SC implanted animals show sporadic and few CST axons caudal to the injury site, a number of PST-GFP SC implanted animals have many CST axons present within the caudal cord for distances beyond 1 mm. Scale bar = 60 μm. Statistical significance indicated at * $p < 0.05$.

Figure 9. Animals receiving PST-GFP SC implants show significant improvements in open-field locomotion and foot placement on the gridwalk test compared to GFP SC and SCI only controls
A. Implantation of GFP SCs produces a modest, but transient, improvement in open-field locomotion as assessed using the BBB score over SCI controls from 4 to 6wk post-SCI. This improvement is further enhanced over GFP SCs, and persisted through endpoint, by the addition of surface PSA, from 5 to 9wk post-SCI. **B.** PST-GFP SCs produced a significant improvement in BBB subscores over SCI only controls from 7 to 9wk post-SCI and GFP SCs, at endpoint. **C.** PST-GFP SCs significantly reduced the number of footfall errors observed on the gridwalk from 4 to 8wk post-implantation compared to both control groups. Statistical significance indicated at * $p < 0.05$ or ** $p < 0.01$ versus SCI only controls; # $p < 0.05$ versus GFP SC controls.

Figure 10. Linear correlation analysis shows significant correlation between SC caudal migration or caudal CST axon growth and improved function on the gridwalk test A–B. While linear correlation analysis showed no significant correlation between caudal SC migration or descending axon growth and BBB scores (See Table 1), significant correlations were observed between caudal SC migration and gridwalk footfall errors (**A**) as well as caudal CST axon growth and gridwalk footfall errors (**B**). An r value of >0.468 was required to demonstrate a significant correlation between outcome measures. For caudal CST axon numbers, values at 1000–1500 μm caudal were used. Statistical significance indicated at $*p<0.05$.

Table 1

Linear Correlation Analysis of Histological and Functional Outcome Measures

Comparative Measures	p Value	Significance
CST axons caudal [#] /Gridwalk footfall errors	0.5712	*p<0.05
SC caudal migration distance/Gridwalk footfall errors	0.4705	*p<0.05
CST axons caudal [#] /BBB Score	0.4201	NS
SC caudal migration distance/5HT axons caudal [#]	0.4188	NS
SC caudal migration distance/CST axons caudal [#]	0.3539	NS
SC caudal migration distance/BBB Score	0.2755	NS
5HT axons caudal [#] /Gridwalk footfall errors	0.1673	NS
SC caudal migration distance/BBB Subscore	0.1471	NS
5HT axons caudal [#] /BBB Score	0.0357	NS
CST axons caudal [#] /BBB Subscore	0.0291	NS
5HT axons caudal [#] /BBB Subscore	0.0046	NS

[#] Caudal axon presence measured at 1000–1500 µm from the caudal lesion: host cord interface