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Research report

Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord

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Abstract

Neural precursor cells (NPCs) are promising grafts for treatment of traumatic CNS injury and neurodegenerative disorders because of their potential to differentiate into neurons and glial cells. When designing clinical protocols for NPC transplantation, it is important to develop alternatives to direct parenchymal injection, particularly at the injury site. We reasoned that since it is minimally invasive, intrathecal delivery of NPCs at lumbar spinal cord (lumbar puncture) represents an important and clinically applicable strategy. We tested this proposition by examining whether NPCs can be delivered to the injured cervical spinal cord via lumbar puncture using a mixed population of neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs). For reliable tracking, the NPCs were derived from the embryonic spinal cord of transgenic donor rats that express the marker gene, human placental alkaline phosphatase, under the control of the ubiquitous Rosa 26 promoter. We found that mixed NRP/GRP grafts can be efficiently delivered to a cervical hemisection injury site by intrathecal delivery at the lumbar cord. Similar to direct parenchymal injections, transplanted NRP/GRP cells survive at the injury cavity for at least 5 weeks post-engraftment, migrate into intact spinal cord along white matter tracts and differentiate into all three mature CNS cell types, neurons, astrocytes, and oligodendrocytes. Furthermore, very few graft-derived cells localize to areas outside the injury site, including intact spinal cord and brain. These results demonstrate the potential of delivering lineage-restricted NPCs using the minimally invasive lumbar puncture method for the treatment of spinal cord injury.

Theme: Development and regeneration
Topic: Transplantation

Keywords: Neural stem cell; Spinal cord injury; Neuron; Glial cell; Transplantation; Neural progenitor

1. Introduction

Transplantation of neural precursor cells (NPCs) is a promising therapeutic strategy for the treatment of CNS injuries and neurodegenerative disorders [15]. Transplanted cells offer a number of possible therapeutic uses, including delivery of therapeutic factors to provide trophic support or missing gene products, mobilization of endogenous NPCs and replacement of lost or dysfunctional cells [36]. Traumatic spinal cord injury (SCI) can specifically benefit from the engraftment of NPCs. Transplanted cells may remyelinate denuded axons, decrease glial scar formation, prevent secondary cell loss, promote regeneration, form bridges and relays, and replace neural cells. A number of studies have demonstrated that transplanted NPCs promote anatomical plasticity and modest behavioral recovery in contusive and surgical lesion models of SCI [25,28]. The vast majority of studies have employed parenchymal injection of cells directly into the lesion site. This strategy is designed to efficiently deliver cells into the injury site; however, if transferred to the

Abbreviations: AP human placental alkaline phosphatase transgene; GRP glial-restricted precursor; NPC neural precursor cell; NRP neuronal-restricted precursor; SCI traumatic spinal cord injury

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clinic, this would be an expensive and invasive technique requiring general anesthesia and risking damage to the already injured spinal cord. In addition, many diseases that are candidates for NPC transplantation therapy are diffuse (e.g., multiple sclerosis) and would require delivery of engrafted cells over extensive, non-contiguous areas [34]. It is therefore important to develop alternative NPC delivery methods to direct and local parenchymal injections.

Transplantation of NPCs into the cerebrospinal fluid offers a potential alternative to intraparenchymal injection. Previous work has demonstrated that NPCs and bone marrow stromal cells can be delivered to SCI sites [5,29,30,41] and MS plaques [9,14,34] via intraventricular injection, and can promote behavioral improvement. Intrathecal injection [11,12] at lumbar levels (lumbar puncture; spinal tap) is a very promising strategy because of its minimal invasiveness, simplicity, and low cost, and because it would allow multiple injections at desired intervals. NPCs may be well suited for lumbar puncture delivery because of their responsiveness to signals expressed in the injured CNS. Previous work has shown that endogenous NPCs not only proliferate and differentiate following injury [18,20,42], but both endogenous [4,27,31,32] and grafted NPCs [2,37,38] can migrate to sites of CNS pathology.

The objective of the present study was to assess whether NPCs can be successfully delivered to the injured cervical spinal cord via lumbar puncture transplantation. This will aid in determining promising NPC engraftment protocols for the treatment of SCI. Specifically, a mixed population of neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs) – NPCs with lineage restrictions for neurons and glia, respectively – was used in this study [24,35]. Transplanted cells were derived from embryonic day-13.5 fetal spinal cord of transgenic donor rats that express the marker gene, human placental alkaline phosphatase, under the control of the ubiquitous Rosa 26 promoter [22,26] for reliable graft tracking. Previous work has demonstrated that NRPs and GRPs, whether grafted individually or together, display robust survival, extensive migration, and differentiation following transplantation into the intact [10,17,19,23,43] CNS. However, while mixed NRPs/GRPs grafted into the injured spinal cord show the same properties, NRPs grafted alone show poor survival and differentiation [18,23]. Furthermore, NRP/GRP grafts promote recovery in motor, sensory, and autonomic functions in the thoracic contusion model of SCI (Mitsui et al., unpublished data). However, all these previous studies involved direct parenchymal transplantation of cells.

In this study, we report that mixed NRP/GRP grafts can be efficiently delivered to a cervical lateral funiculotomy injury site via intrathecal delivery at the lumbar cord. Similar to direct parenchymal injections, transplanted NRP/GRP cells survive in the injury cavity for up to 5 weeks post-engraftment (longest time point examined), migrate into intact spinal cord along white matter tracts, morphologically mature and differentiate into all three mature CNS cell types, neurons, astrocytes, and oligodendrocytes. Furthermore, very few graft-derived cells localize to areas outside the injury site, including intact spinal cord and brain. These results demonstrate that mixed populations of lineage-restricted precursor cells can be delivered to the injured spinal cord via clinically attractive delivery methods.

2. Materials and methods

2.1. Neural precursor cultures

2.1.1. Isolation and culturing of NRPs and GRPs

NRPs and GRPs were isolated from the embryonic day-13.5 spinal cord of transgenic Fischer 344 rats that express the marker gene, human placental alkaline phosphatase (AP), under the ubiquitous Rosa 26 promoter. The use of AP transgenic animals to derive NPCs allows for unambiguous detection of transplanted cells in the CNS. Briefly, embryos were isolated in a dish containing DMEM/F12. Trunk segments were incubated in a collagenase Type I (10 mg/mL)/dispase II (20 ng/mL)/HBSS solution for 8 min at room temperature to allow for peeling away of meninges from the cords. Cords were dissociated using a 0.05% trypsin/EDTA solution for 20 min at 37 °C. Cells were then plated in NRP complete medium [(DMEM-F12, BSA (1 mg/mL; Sigma; St. Louis, MO), B27 (Invitrogen), bFGF (20 ng/mL; Peprotech; Rocky Hill, NJ), Pen-Strep (100 IU/mL; Invitrogen), N2 (10 μL/mL; Invitrogen); bFGF (10 μg/mL) and NT-3 (10 μg/mL; Peprotech)] on poly-L-lysine-coated (13.3 μg/mL; Sigma) and laminin-coated (20 μg/mL; Invitrogen) dishes.

2.1.2. Preparation of cells for grafting

Although we have previously shown that NRPs and GRPs can be individually isolated, we reasoned that, for traumatic spinal cord injury, we will need replacement of both neurons and glia. Following embryonic dissection, NRPs and GRPs were co-cultured for 5–10 days prior to transplantation. The mixed population of NRPs and GRPs was dissociated from culture flasks using 0.05% trypsin/EDTA, washed and re-suspended at a concentration of 100,000 cells/μL (in basal media) for transplantation. Cells were placed on ice throughout the grafting session. After the completion of the grafting session, cell viability was assessed using the trypan blue assay. Viability was always found to be greater than 90%. The composition of the NRP/GRP cultures, with respect to the presence of undifferentiated NPCs and the absence of mature cells, was verified before grafting by staining for the immature neural marker nestin, for markers of mature neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (RIP), as well as for markers of NRPs (E-NCAM) and GRPs (A_2B_5).

2.2. Animal surgery and transplantation

2.2.1. Adult spinal cord injury

Lateral funiculus injuries were created at the cervical 4 spinal cord level. Adult female Sprague–Dawley rats (approximately 250 g) received intraperitoneal injections of anesthetic cocktail [acepromazine maleate (0.7 mg/kg; Fermenta Animal Health, Kansas City, MO), ketamine (95 mg/kg; Fort Dodge Animal Health; Fort Dodge, IA), and xylazine (10 mg/kg; Bayer, Shawnee Mission, KS)]. The back musculature was excised, and a laminectomy was performed at the C3/4 level. The dura was incised above the dorsal root entry zone. Microscissor cuts were created at the rostral and caudal extents of the injury. Aspiration was used to selectively ablate only the lateral white matter tracts, as well as a minimal portion of the dorsal and ventral gray matter. The dorsal columns and central canal were unaffected. Once hemostasis was achieved, Type I Collagen matrix (Vitrogen) or a hydrogel matrix was implanted into the injury cavity using a 10-μL Hamilton Gastight syringe (Hamilton; Reno, NV) or forceps, respectively. The matrices completely filled the cavities. Dura was closed with 9-0 suture; muscle was re-apposed; skin was closed with wound clips. Animals received Bupranorphine (analgesic employed to minimize pain associated with the surgery) and methylprednisolone (Pharmacia and Upjohn; Kalamazoo, MI) postoperatively.

2.2.2. Lumbar puncture delivery of cells and beads

A mixed population of NRPs and GRPs (1×10^6 cells or 4×10^6 cells in 40 μL of basal media) or fluorescent beads (1×10^6 beads in 40 μL of basal media) was transplanted by intrathecal delivery in the lumbar cord. Two cell delivery paradigms were employed. In the first, 1×10^6 cells were grafted by lumbar puncture only once 24 h post-injury ($n = 9$). In the second, cells were delivered three times (4×10^6 cells per injection) via lumbar puncture at 1-week intervals, beginning 7 days after injury ($n = 8$). Fluorescent beads were delivered with a single injection 1 week post-injury ($n = 3$). Adult female Sprague–Dawley rats (approximately 250 g) received inhalation (Isoflurane) anesthesia. Briefly [6], the rat was anesthetized and placed on an operating surface that flexes the animal's back. A small (1 cm) longitudinal incision was then made over the L3–5 spinous processes, and the skin was retracted. A neonatal LP needle (25-gauge) was advanced into the spinal canal at L3–4 or L4–5. Proper placement of the needle in the lumbar intrathecal space was indicated by three signs: (1) a feeling of "give" at the time of entry (soft sign); (2) a tail flick (more definitive sign); and (3) presence of CSF in the needle hub (most definitive sign). Once proper needle placement was confirmed, the CSF present in the needle hub was aspirated using a micropipette, and cells were injected into the CSF during a 30-s period. Additionally, 10 μL of saline was used to flush the needle, and the LP needle was withdrawn after replacing the stillette. The skin was closed with wound clips. Animals were immunosuppressed by subcutaneous administration of cyclosporine A (10 mg/kg; Sandoz Pharmaceuticals, East Hanover, NJ) daily beginning 3 days before grafting and continuously until sacrifice to avoid rejection of engrafted cells. The care and treatment of animals in all procedures was conducted in strict accordance with the guidelines set by the *NIH Guide for the Care and Use of Laboratory Animals* and the Drexel University IACUC.

2.3. Tissue processing

Animals were sacrificed at various time points (4 days, 2 weeks, and 5 weeks following final cell delivery session) following transplantation by transcardial perfusion with 0.9% saline, followed by ice-cold 4% paraformaldehyde (Fisher Scientific; Pittsburgh, PA). Spinal cord and brain were removed from the animal, followed by cryoprotection in 30% sucrose (Fisher)/0.1 M phosphate buffer at 4 °C for 3 days. The tissue was embedded in OCT (Fisher), fast frozen with dry ice, and stored at −80 °C until processed. Spinal cord tissue blocks were cut in the sagittal or transverse plane at 20 μm thickness, while brain tissue blocks were cut either in the coronal or sagittal plane at a thickness of 30–40 μm. Sections were collected on gelatin- and poly-L-lysine-coated glass slides and stored at −80 °C until analyzed. Subsets of spinal cord and brain slices were collected in PBS for free-floating histochemistry.

2.4. Alkaline phosphatase histochemistry

Serial sections were analyzed by alkaline phosphatase histochemistry to assess the presence, location, migration, and morphology of graft-derived cells in the spinal cord and striatum. Sections were washed with PBS, heated at 60 °C for 1 h to inactivate endogenous enzyme activity, washed briefly in alkaline phosphatase buffer [(Tris; 100 mM; Fisher), (NaCl; 100 mM; Fisher), (MgCl2; 50 mM; pH 9.5; Fisher)], and finally incubated at room temperature in the dark with alkaline phosphatase staining solution [(NBT; 1.0 mg/mL; Sigma), (BCIP; .1 mg/mL; Sigma), (levamisole; 5 mM; Sigma) in alkaline phosphatase buffer] for 1.5–2.0 h. Slides were cover-slipped in hard-set Vectashield (Vector; Burlingame, CA) and visualized with light microscopy.

2.5. Immunohistochemistry/phenotypic analysis

Tissue sections and cultured cells were washed in PBS, blocked in 10% goat serum (Invitrogen) for 1 h at room temperature and incubated with primary antibody solution at 4 °C overnight. Both monoclonal and polyclonal (1:200; Accurate, Westbury, NY) antibodies against human placental alkaline phosphatase were used to identify graft-derived cells. A number of primary antibodies were used to assess the phenotype of cells. Nestin (1:1000; monoclonal; Pharmingen; San Diego, CA) was used to identify

undifferentiated NPCs (NEP cells, NRPs and GRPs). NRPs were identified specifically using E-NCAM (1:200; Chemicon; Temecula, CA), while GRPs were identified using A_2B_5 (1:500; Chemicon). Neurons were identified using the antibodies: MAP2 (1:100; monoclonal; Chemicon); NeuN (1:100; monoclonal; Chemicon). Astrocytes were identified using GFAP (1:100; monoclonal; Chemicon). Oligodendrocytes precursors were identified using the antibodies: NG2 (1:250; polyclonal; Chemicon), Sox-10 (1:200; polyclonal; Chemicon). Mature oligodendrocytes were identified with RIP (1:1000; monoclonal; Chemicon). Macrophages/reactive microglia were identified with ED1 (1:500; monoclonal; Accurate) and OX42 (1:500; monoclonal; Accurate). Samples were incubated for 2 h at room temperature with goat anti-mouse and goat anti-rabbit secondary antibodies (1:200; Jackson, West Grove, PA) conjugated to rhodamine or FITC. Samples were counterstained with DAPI (1:1000; Sigma) to identify nuclei, and cover-slipped with anti-fade mounting media (Fluorosave, CN Biosciences; La Jolla, CA). Slides were subsequently stored at −20 °C. For double immunofluorescence staining of AP-positive cells and lineage-specific markers, regions with relatively small numbers of cells were chosen for imaging and presentation to achieve good morphological definition and to avoid the difficulties associated with large clustering. Images were acquired on either a Leica DMRBE fluorescence microscope (Leica Microsystems; Bannockburn, IL) using a Photometric Sensys KAF-1400 CCD camera (Roper Scientific; Trenton, NJ) or on a Leica TCS SP2 laser confocal microscope (Leica). Images were analyzed using either IP Lab (Scanalytics; Fairfax, VA) or Leica confocal software version 2.0 (Leica). Adobe Photoshop CS (Adobe, San Jose, CA) was used to prepare figures.

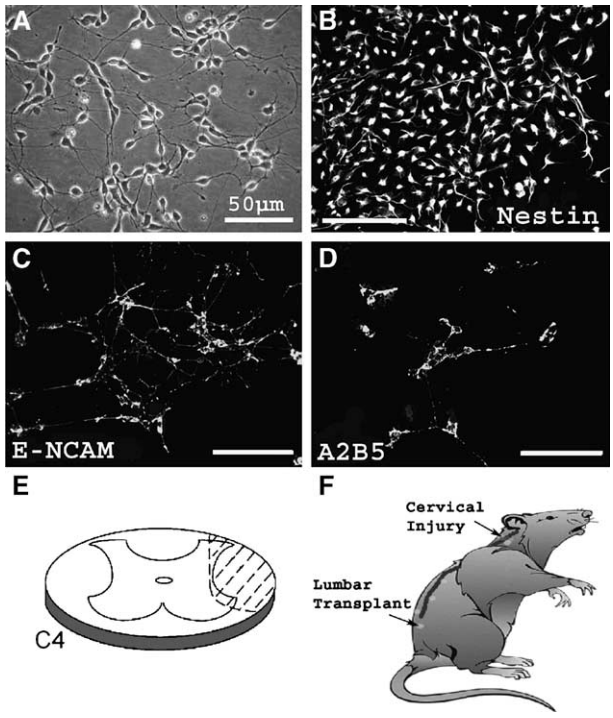

Fig. 1. In vitro characterization of the grafted cells and experimental paradigm. Prior to transplantation, mixed NRP/GRP cells were characterized to determine their morphological properties and antigenic profile. The mixed NRP/GRP cultures consisted of only small phase-bright bipolar cells (A), and all cells expressed the early neural marker, nestin (B). In addition, cells in the mixed NRP/GRP cultures expressed either E-NCAM (C) or A_2B_5 (D), markers of NRPs and GRPs, respectively. A lateral funiculus injury was created at the cervical-4 spinal cord level (E). This type of injury results in selective ablation of the lateral white matter tracts, as well as a minimal portion of the dorsal and ventral gray matter. The dorsal columns and central canal were unaffected. No cells were implanted at the time of injury, only type-1 collagen matrix (Vitrogen) or hydrogel matrix. Cells were non-invasively transplanted via intrathecal delivery at the lumbar cord (F) at various time points post-injury. All scale bars represent 50 μm.

3. Results

3.1. In vitro characterization

NRPs and GRPs derived from embryonic spinal cords of transgenic AP rats were co-cultured for 5–10 days following dissection, and remained as undifferentiated lineage-restricted precursors. During the culturing period in the non-differentiation media, the cells showed the characteristic morphology of small, phase-bright, lineage-restricted precursors (Fig. 1A). We found that at the time of grafting, all cells in the NRP/GRP cultures expressed the early neural marker, nestin (Fig. 1B), confirming that they remained at the NPC stage. The cultured NRP/GRP cells did not express mature markers of neurons (NeuN), astrocytes (GFAP), or oligodendrocytes (RIP, NG2, and Sox-10) (data not shown), confirming again that cultures did not contain differentiated cell types. Consistent with this conclusion, the cultured cells expressed either E-NCAM (Fig. 1C) or A_2B_5 (Fig. 1D), markers of NRPs and GRPs, respectively. Together, these results strongly indicate that the cultures were composed almost exclusively of lineage-restricted precursors, both NRPs and GRPs, and were devoid of multipotent neural stem cells and mature cells types.

3.2. Localization of NRP/GRP grafts

We sought to determine whether NRP/GRP grafts could be delivered to a cervical spinal cord injury site (Fig. 1E) via intrathecal delivery of cells at the lumbar cord (Fig. 1F). Three transplantation paradigms were employed in this study in an effort to establish consistent delivery of neural precursor cells by varying parameters such as the number of cells grafted, the time of delivery, and the number of cell injections. In addition, delivery of fluorescent-labeled beads was used as a control for the specificity of the cells to reach and accumulate at the injury site. In the first experiment, 1.0×10^6 NRP/GRP cells were transplanted via lumbar puncture at a single time point 24 h after injury. In the

second experiment, three doses of 4.0×10^6 NRP/GRP cells were delivered at 1-week intervals, beginning 1 week post-injury. Finally, in the third experiment, a single dose of 1.0×10^6 fluorescent-labeled beads was transplanted 1 week post-injury.

3.2.1. Spinal cord

Graft-recipient animals were sacrificed at 4 days, 2 weeks, and 5 weeks following the final transplantation session to determine the profile of cell survival at both early and late time points. Serial sections were assessed by alkaline phosphatase (AP) histochemistry to detect the presence of labeled cells in vivo. In both lumbar puncture grafting paradigms, transplanted NRP/GRP cells were found in the injury site of all recipient animals, despite the long distance between the injection site (lumbar cord) and injury site (cervical cord). Cells were localized throughout the injury site (Figs. 2A–C, 3A), but were concentrated in greatest numbers at the interface of the CSF and injury site (Fig. 2B). Grafted cells were detected in the injury site as long as 5 weeks post-transplantation (longest time point examined). When comparing the efficiency of NRP/GRP delivery in the different experiments, greater numbers of transplanted cells were found in the injury site following the multiple injection protocol (Figs. 3A–F) relative to a single dose (Figs. 2A–D). Transplant-derived cells were found at the spinal cord injury site, but not in adjacent intact spinal cord tissue (Figs. 2C, 3A) or at other segments of spinal cord (data not shown). These results show that lineage-restricted neural precursors, NRPs and GRPs, can be delivered to the injured adult spinal cord via relatively non-invasive intrathecal transplantation at the lumbar cord, with increased efficiency observed with multiple cell injections and increased cells grafted.

3.2.2. Brain

In addition to the spinal cord, the brains of graft recipients were examined to further characterize the presence of transplanted cells in the brain traveling through the CSF. The analysis showed that the NRP/GRP cells delivered via the lumbar spinal cord were found sparsely at various levels of the brain such as the hippocampus, striatum (Fig. 4A—inset) and cerebellum, only in areas lining the ventricular system. Furthermore, grafted cells did not penetrate the parenchyma of the brain (Fig. 4A); instead, they were located at the interface of the ventricles and the parenchyma. However, these cells survived up to 5 weeks, and displayed very mature morphologies, including process extension (Fig. 4A—inset).

3.3. Morphology and migration of the graft

Cells delivered via lumbar puncture migrated out of the injury site into the adjoining intact spinal cord along white matter pathways in both the rostral (Fig. 3B) and caudal (Fig. 3C) direction up to distances of 10 mm away from the injury site (Fig. 3D). Migrating cells often adopted an immature bipolar migratory morphology (Fig. 3E). NRP/GRP cells delivered by lumbar puncture also elaborated mature morphologies within the injured (Fig. 2D) and

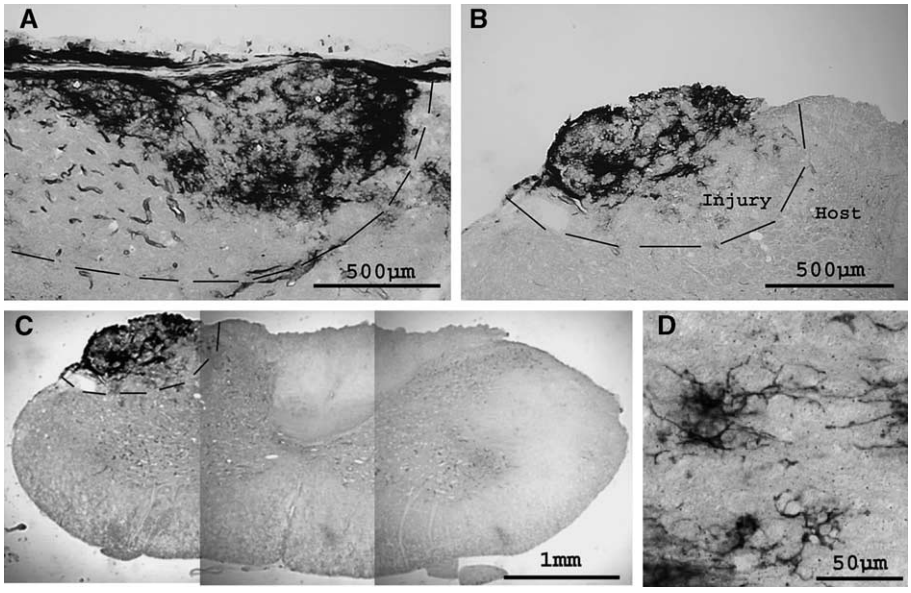

Fig. 2. Lumbar puncture transplantation of NRP/GRP cells: single delivery. A mixed population of NRPs and GRPs was delivered to the injured spinal cord by intrathecal injection at the lumbar cord 24 h post-injury. Alkaline phosphatase (AP) histochemistry was used to detect the presence of the transplanted cells. Grafted cells were present throughout the injury site at both 3 (A) and 5 (B) weeks following transplantation. Morphological maturity of grafted cells is seen at both 3 (D) and 5 weeks post-transplantation. The presence of grafted cells can be noted at the injury site, but not in the surrounding uninjured tissue (C). The dashed lines in each panel represent the interface between host spinal cord and the injury site. All panels are transverse sections, with the dorsal aspect facing upwards, except (A), a sagittal section. Scale bars represent 500 μm (A, B), 1 mm (C), or 50 μm (D).

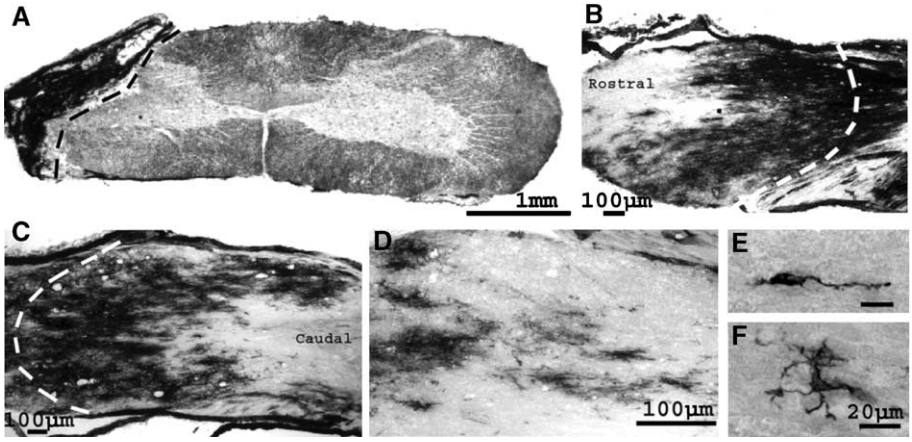

Fig. 3. Lumbar puncture of NRP/GRP cells: multiple deliveries. A mixed population of NRPs and GRPs was delivered to the injured spinal cord by intrathecal injection at the lumbar cord at multiple time points post-injury. Alkaline phosphatase (AP) histochemistry was used to detect the presence of the transplanted cells. Grafted cells are present throughout the injury site at 5 weeks (A) following the final transplantation session. Grafted cells migrated out of the injury site along white matter tracts of the intact spinal cord in both rostral (B) and caudal (C) directions to distances up to 1 cm (D). The dashed lines in each panel represent the interface between host spinal cord and the injury site. Sections are in the transverse (A) or sagittal (B–F) planes, with the dorsal aspect facing upwards. Scale bars represent 1 mm (A), 100 μm (B–D), or 20 μm (E, F).

intact spinal cord (Fig. 3F), as well as in the brain (Fig. 4A—inset).

3.4. Inflammatory cells at the site of injury

To assess the presence of infiltrating macrophages/reactive microglia at the injury site, we carried out double-immunofluorescence staining for the AP transgene and markers of macrophages/reactive microglia, ox42 (Figs. 5A and B) and ED1 (Figs. 5C–G). The analysis showed the paucity of inflammatory cells present in the injury site following lumbar puncture delivery of NRP/GRP cells, suggesting that the detection of graft-derived cells is not a product of trapping or cell fusion with inflammatory elements.

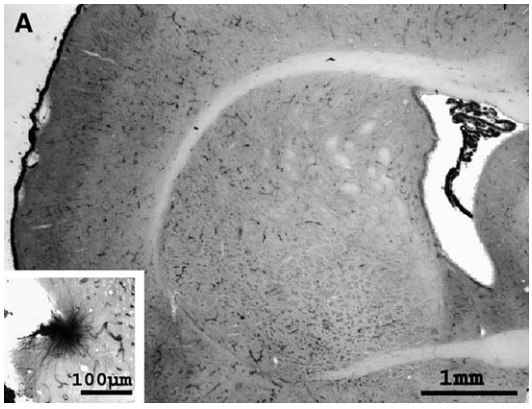

Fig. 4. Localization of NRP/GRP cells in the brain. Alkaline phosphatase (AP) histochemistry was used to detect the presence of the transplanted cells in the brains of lumbar puncture recipients. At all levels of the brain, grafted cells were not present deep in the parenchyma (A). Graft cells were only found directly adjacent to the ventricular system at 3 and 5 weeks following the final transplantation session (inset). Graft-derived cells elaborated mature morphologies such as process extension. Sections are in the coronal plane (A and inset), with the dorsal aspect facing upwards. Scale bars represent 1 mm (A) or 100 μm (inset).

3.5. Localization of fluorescent-labeled beads

Similar to NRP/GRP cells, fluorescent-labeled polystyrene beads were delivered by lumbar puncture injection and served as a control for specificity. The inert polysterene beads possess a diameter similar to the NRPs and GRPs, and

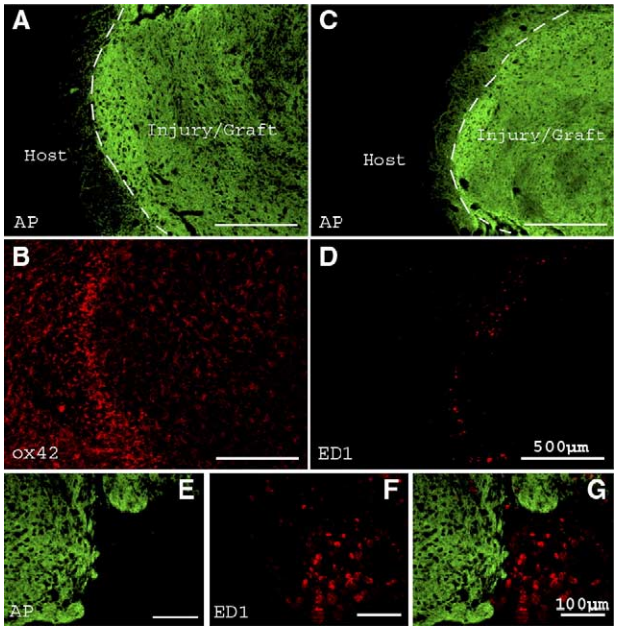

Fig. 5. Inflammatory cell infiltration at the injury site. Double immunofluorescence staining for alkaline phosphatase (A, C, and E, green) and markers of macrophages/reactive microglia (ox42; B, red) (ED1; D and F, red) shows the paucity of inflammatory cells present in the injury site following lumbar puncture delivery of NRP/GRP cells (G, overlay). The dashed lines in each panel represent the interface between host spinal cord and the injury/graft site. Sections are in the transverse plane, with the dorsal aspect facing upwards. Scale bars represent 500 μm (A–D) or 100 μm (E–G).

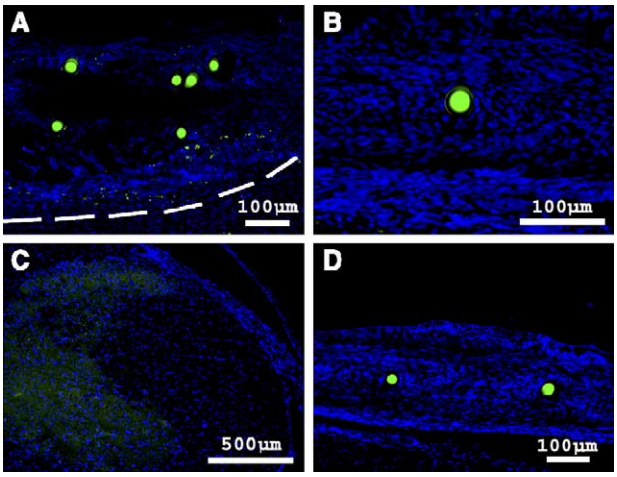

Fig. 6. Lumbar puncture delivery of fluorescent beads. Fluorescent-labeled polystyrene beads were delivered to the injured spinal cord by intrathecal injection at the lumbar cord 7 days post-injury. Transplanted beads were predominantly found in the parenchyma of the injury site (A and B). However, in comparison to the large numbers of graft-derived cells found in the injury site, very few beads localized to the injury cavity. Beads were not detected in the parenchyma of the brain (data not shown) or intact spinal cord (C), but they were found attached to the meninges surrounding both the intact and injured spinal cord (D). Sections are in the sagittal (A, B, and D) or transverse (C) plane, with the dorsal aspect facing upwards. Scale bars represent 100 μm (A, B, and D) or 500 μm (C).

their engraftment was employed to determine if NRP/GRP cells localize to the site of injury by a non-specific mechanism (i.e., diffusion and attachment) or whether a selective mechanism is involved in cell distribution and accumulation at the SCI site (i.e., adhesion). In contrast to the large number of cells observed with the NRP/GRP grafts, very few beads were found at the injury cavity (Figs. 6A and B). In addition, no beads were detected in the parenchyma of the brain (data not shown) or intact spinal cord (Fig. 6C); instead, they were found loosely attached to the meninges surrounding both the intact and injured spinal cord (Fig. 6D).

3.6. Phenotypic fate of the grafts

Although the AP transgene is a very reliable and stable marker for detection of grafted cells, it does not allow an unambiguous identification of neuronal and glial phenotypes. The phenotypic fate of the NRP/GRP grafts following transplantation was therefore assessed by co-localization of the AP transgene with specific markers of mature CNS cell types. We found that NRP/GRP grafts delivered via lumbar puncture differentiated into neurons, astrocytes, and oligodendrocytes, along with expression of synaptic markers by graft cells, within the injury site. The figures illustrate regions with relatively small numbers of cells to show good morphological definition and to avoid the difficulties associated with large clustering; however, the images represent the phenotypic fate of transplant-derived cells throughout the injury site.

3.6.1. Neuronal differentiation

The challenge of delivering NPCs to the injured spinal cord via lumbar puncture transplantation is particularly great for getting neuronal precursors to differentiate into neurons. Previous work has shown that when NRPs are grafted into the injured spinal cord, they show minimal differentiation into neurons [10]. Our studies with mixed NRPs/GRPs have demonstrated that together these cells can produce both glia and neurons following transplantation in the intact or injured adult CNS [23]. We therefore sought to determine whether NRPs, delivered by lumbar puncture to the injured spinal cord, can also produce a similar differentiation

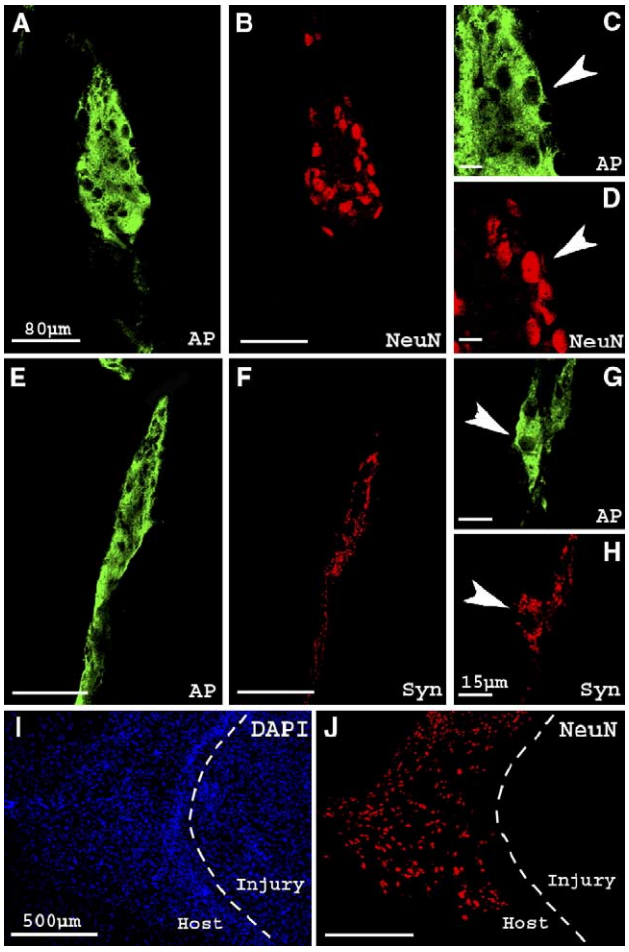

Fig. 7. Mixed NRP/GRP grafts differentiate into neurons and express synaptic markers in the injured spinal cord following lumbar puncture delivery. In the injured spinal cord, mixed NRP/GRP grafts differentiated into mature neurons. Confocal analysis of grafted cells by double immunofluorescence staining for alkaline phosphatase (A and C, green) and NeuN (B and D, red) shows neuronal differentiation of graft-derived cells in the injured spinal cord. NRPs/GRPs also expressed synaptic markers. Confocal analysis of double immunofluorescence staining for alkaline phosphatase (E and G, green) and synaptophysin (F and H, red) suggests the possible association of grafted cells with synapse formation in the injured spinal cord. In the absence of NRP/GRP grafts, mature neurons are not found in the injury site, (DAPI, I, blue; NeuN, J, red). The dashed lines in each panel represent the interface between host spinal cord and the injury site. All panels are in the transverse plane. Arrowheads denote double-labeled cells. Scale bars represent 80 μm (A, B, E, and F), 15 μm (C, D, G, and H), or 500 μm (I, J).

profile. Neuronal differentiation was demonstrated by co-localization of the AP transgene with NeuN (Figs. 7A–D) or MAP2 (data not shown), markers of mature neurons. In addition to neuronal maturation, graft-derived cells expressed markers of synapse formation, as demonstrated by the co-localization of the AP transgene and the synaptic vesicle protein, synaptophysin (Figs. 7E–H).

3.6.2. Glial differentiation

Previous work has shown that GRPs differentiate into astrocytes and oligodendrocytes, but not neurons, in the intact and injured adult CNS [18,23]. Again, we wanted to determine if this same pattern of differentiation occurs in the injured spinal cord after lumbar puncture transplantation. We found that mixed NRP/GRP grafts differentiated into both types of CNS glial cells, astrocytes and oligodendrocytes, in the injured spinal cord following lumbar puncture delivery. The astrocytic phenotype was assessed by co-localization of AP and GFAP (Figs. 8A–D), a marker of mature astrocytes, while graft-derived oligodendrocytes were identified by co-localization of AP and RIP (Figs. 8E–H), a marker of mature oligodendrocytes.

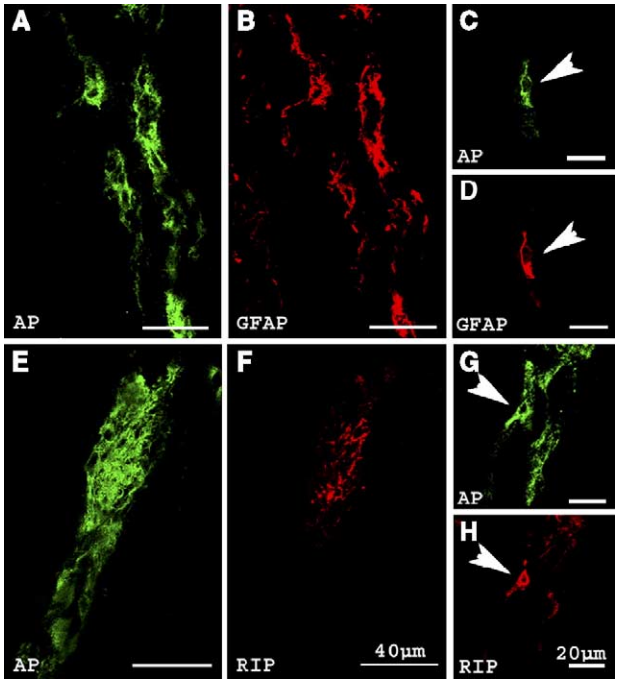

Fig. 8. Mixed NRP/GRP grafts differentiate into astrocytes and oligodendrocytes in the injured spinal cord following lumbar puncture delivery. In the injured spinal cord, mixed NRP/GRP grafts differentiated into mature astrocytes. Confocal analysis of grafted cells by double immunofluorescence staining for alkaline phosphatase (A and C, green) and GFAP (B and D, red) shows astroglial differentiation of graft-derived cells in the injured spinal cord. NRPs/GRPs also differentiated into mature oligodendrocytes. Confocal analysis of double immunofluorescence staining for alkaline phosphatase (E and G, green) and RIP (F and H, red) shows oligodendoglial differentiation of graft-derived cells in the injured spinal cord. All panels are in the transverse plane. Arrowheads denote double-labeled cells. Scale bars represent 40 μm (A, B, E, and F) or 20 μm (C, D, G, and H).

4. Discussion

Developing effective protocols for the transplantation of NPCs is a promising therapeutic strategy for repairing the CNS not only in the traditional sense of cell replacement but also by providing trophic support and protection. Ideally, differentiated neurons could contribute to plasticity and formation of new circuits, oligodendrocytes could participate in remyelination, and new astrocytes could reduce the extent of cell death and injury. Thus, effective transplantation of NPCs has a potential for treating CNS injury and neurodegenerative disorders. As these therapies are getting ready for clinical application, it is important to address practical issues related to NPC transplantation. These include timing, location and method of cell delivery, cost concern, reproducibility, and minimization of the additional trauma associated with the grafting procedures. Based on these concepts, our study has been designed to develop a strategy that considers both therapeutic and practical issues in achieving an optimal procedure for transplantation of NPCs.

To start with, an important requirement for reliable grafting experiments is the ability to follow the cells with respect to survival, migration, and fate. Labeling cells with dyes is notoriously problematic because of possible leakage, while various vectors used for introducing marker transgenes often exhibit down-regulation in the CNS. Thus, the availability of the AP transgene, expressed under the control of the ubiquitous Rosa 26 promoter [22,26], allowed us to isolate and graft NPCs and then detect them unambiguously within the host to assess their properties. The phenotypic analysis of grafted cells was also facilitated both by the presence of relatively few immune cells at the site of injury and the ability of engrafted cells to migrate out of the site of injury.

Our working hypothesis for the intrathecal delivery of NPCs has been that cells can enter the CNS parenchyma when the outer lamina is disrupted. We therefore reasoned that there will be a window of therapeutic opportunity to inject the cells away from the injury site to avoid the invasive and traumatic steps associated with direct grafting. This hypothesis also predicts that the intrathecal delivery procedure will be effective in acute spinal cord injury but not in conditions or locations where the outer basal lamina is intact. Indeed, we found that the grafted NPCs were present predominantly at the site of injury and not in the intact spinal cord or brain. The hypothesis is also supported by the finding that the fluorescent-labeled beads, which serve as biologically-inert control grafts, were also able to localize to the site of injury and not to intact tissue. While the physical disruption of the spinal cord tissue and the surrounding meninges remains the most straightforward explanation for the ability of the grafted cells to reach the injury cavity, chemotactic signaling and adhesion molecules are likely to play a role. This idea is supported by the presence of very few beads at the site of injury in comparison to transplanted

cells. We therefore suggest that some biological mechanism may also participate in the localization process of NRPs and GRPs to the injured spinal cord. Presumably, factors produced by injured cells and invading immune cells could contribute to the NPC response by attracting and retaining the NPCs. In vitro studies have demonstrated that NPCs migrate in response to cells and factors found at the SCI site [7,40], including microglia [1], chemokines [13,21,33,39], inflammatory cytokines such as TNFα and IFNγ [8], and PDGF [3,16]. Such a signaling effect could be exploited in NPC-based therapy for specific delivery of the cells from remote locations. There may also be adhesion molecules and connective tissue produced at the site of injury which serve as a permissive matrix for the grafted cells. Taken together, the results of this study support the conclusion that both physical and biological mechanisms may be operating to localize transplanted NRPs and GRPs to the spinal cord injury site. Regardless of the precise mechanism, our data have shown that the inflammation process at the damaged environment does not prevent the NPCs from integrating into the site of injury and thus extending rather than closing the window of therapy.

We have shown that mixed populations of lineage-restricted precursors derived from the fetal spinal cord can be delivered to a cervical spinal cord injury site via intrathecal delivery at the lumbar cord. This technique provides an easy, quick, and efficient method by which grafted NPCs can be delivered to the traumatically injured spinal cord. Furthermore, in the lumbar puncture procedure, cells are delivered from a remote uninjured location, without triggering additional tissue damage associated with direct parenchymal injection at the site of injury or causing any apparent behavioral abnormalities. In this study, we have also demonstrated that NRPs and GRPs can be delivered to the injured spinal cord by multiple grafting procedures at weekly intervals, suggesting the possibility of dose escalation and titration for individual patients. This strategy may allow for more efficient delivery of cells, as well as flexibility in the temporal design of transplantation protocols, according to the characteristics of the specific pathology. NPCs could also be delivered by this method to other spinal cord and brain injury models. Delivery methods such as lumbar puncture are particularly attractive for more diffuse pathologies. Indeed, Pluchino and colleagues [34] have demonstrated that NPCs, delivered by intraventricular injection, localize to multiple sclerosis plaques and promote behavioral improvement in the EAS mouse model. Work in our laboratory has shown that, using similar protocols, bone marrow stromal stem cells can be delivered to both a cervical lateral funiculus injury [6] and to a thoracic contusion (Bakshi et al., unpublished data) by lumbar puncture transplantation. These findings suggest that the lumbar puncture procedure has a potential for delivery of a variety of stem cells.

The finding that NRPs and GRPs delivered by lumbar puncture localize to the injury site and subsequently differentiate is also important with respect to absence of side effects such as uncontrolled proliferation and dispersion to undesired areas. Using this engraftment technique, transplanted cells were only delivered to the designated region, namely, the injury site. Any localization of cells outside the injury cavity was a product of cell migration. Specifically, graft-derived cells were found to migrate out of the injury site into adjacent intact spinal cord selectively along white matter tracts. This property of NRPs and GRPs is particularly attractive for CNS injury because the migrating cells could potentially support repair and bridging at and around the injury site. The ability of the grafted NRPs and GRPs to differentiate into neurons and glial cells, respectively, at the injury site is the most significant finding with respect to potential therapeutic benefits. For example, NRP-derived neurons may contribute to the rearrangement of host circuits and may promote axonal growth from and through the injury site, thus forming bridges and relays for reconnection of disrupted tracts. Indeed, expression of synaptic markers by the grafted cells is consistent with integration and connectivity with host. The migration stream, consisting mostly of grafted GRPs and subsequently glial cells, could potentially guide and myelinate growing host and graft-derived axons. The astrocytes formed at the injury site could provide trophic support and contribute to neuroprotection.

The finding of both neuronal and glial differentiation suggests that both NRPs and GRPs can effectively be delivered to the injured spinal cord via intrathecal injection at the lumbar cord. The fate of NRPs and GRPs in the injury site, with respect to survival, migration, morphological maturation, and differentiation, is in agreement with previous work that examined the fate of mixed NRP/GRP grafts delivered to the injured cervical spinal cord by direct parenchymal injection [23]. In the present experiments, the survival of transplanted cells was followed for up to 5 weeks post-engraftment; however, more extended survival is predicted based on work showing that NRP/GRP grafts survive for up to 15 months in the adult CNS (Lepore et al., unpublished).

These studies further support the valuable properties of lineage-restricted NPCs for transplantation into the injured spinal cord. Unlike multipotent neural stem cells [23], lineage-restricted NPCs demonstrate robust survival, extensive migration, and efficient neuronal and glial differentiation in both the intact [10,17–19,23,43] and injured [18,23] spinal cord for extended periods of time. Furthermore, ongoing work in our laboratory (Mitsui et al., unpublished data) has shown that mixed grafts of NRPs and GRPs promote recovery of bladder, sensory, and motor functions following transplantation into the contused adult spinal cord. This recovery is associated with anatomical plasticity below the level of the lesion, including increased numbers of 5-HT, CRF, and DBH descending fibers relative to injured animals not receiving transplants, suggesting that grafts promoted sparing and/or sprouting of host fibers.

Future studies will address the regenerative and behavioral efficacy of NRP/GRP grafts delivered via lumbar puncture. These studies must eventually be translated into minimally invasive clinical protocols for repair of spinal cord injury.

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