Cellular therapy after spinal cord injury using neural progenitor cells

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Chapter 3

Adult neural progenitor cells provide a permissive guiding substrate for corticospinal axon growth following spinal cord injury

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ABSTRACT

Adult neural progenitor cells (NPC) are an attractive source for cell transplantation and neural tissue replacement after central nervous system (CNS) injury. Following transplantation of NPC cell suspensions into the acutely injured rat spinal cord NPC survive, however, they migrate away from the lesion site and are unable to replace the injury induced lesion cavity. In the present study we examined 1) whether NPC can be retained within the lesion site after co-transplantation with primary fibroblasts, and 2) whether NPC promote axonal regeneration following spinal cord injury. Co-cultivation of NPC with fibroblasts demonstrated that NPC adhere to fibroblasts and the extracellular matrix produced by fibroblasts. In the presence of fibroblasts, the differentiation pattern of co-cultivated NPC was shifted towards glial differentiation. Three weeks after transplantation of adult spinal cord derived NPC with primary fibroblasts as mixed cell suspensions into the acutely injured cervical spinal cord in adult rats, the lesion cavity was completely replaced. NPC survived throughout the graft and differentiated exclusively into glial cells. Quantification of neurofilament labeled axons and anterogradely labeled corticospinal axons indicated that NPC co-grafted with fibroblasts significantly enhanced axonal regeneration. Both, neurofilament labeled axons and corticospinal axons aligned longitudinally along GFAP expressing NPC derived cells, which displayed a bipolar morphology reminiscent of immature astroglia. Thus, grafted astroglial differentiated NPC promote axon regrowth following spinal cord injury by means of cellular guidance.

1. INTRODUCTION

Cell-based therapies in the injured spinal cord are intended to fill lesion cavities, which typically develop at an injury site, and to provide a cellular growth conducive substrate for re-growing axons. Various cell types such as fibroblasts, olfactory ensheathing cells, Schwann cells and neural stem/progenitor cells have been used to replace injured spinal cord parenchyma, and to elicit axonal regeneration [1-6]. Neural stem/progenitor cells represent a particularly attractive cell type for CNS transplantation, because they allow organotypic cellular replacement, and may have the capacity to establish growth promoting guidance structures, thus recapitulating cell-based axon guidance in the developing CNS. Indeed, time course studies in the early postnatal rat spinal cord demonstrate that immature astrocytes expressing vimentin and GFAP might promote the longitudinal outgrowth of corticospinal axons [7, 8]. In the injured peripheral nervous system, cellular guidance plays a key role in promoting axonal regeneration. Schwann cells build a longitudinally aligned cellular path, guide regenerating axons along their processes, and express cell adhesion molecules and growth factors in appropriate spatial and temporal sequences [9].

Adult neural progenitor cells (NPC), isolated from the subventricular zone or spinal cord, have been shown to self-renew, and to be multipotent *in vitro* and after transplantation into the CNS [10, 11]. Following transplantation of adult NPC into the intact and injured spinal cord, only astroglial and oligodendroglial differentiation is observed [12-14]. Even after transplantation into the acutely injured spinal cord lesion site, adult NPC survive. However, grafted NPC migrate completely into the surrounding host parenchyma, thus preventing the replacement of the lesion cavity [14]. Therefore, studies investigating the transplantation of NPC in the injured CNS have not been able to determine whether adult NPC promote axonal regeneration across cystic lesion defects caused by spinal cord injury.

Recently, it has been demonstrated in an ischemia model that an additional supporting scaffold such as an artificial biodegradable polymer is required to retain transplanted neural stem cells within lesion cavities developing after ischemia [15]. Fibroblasts naturally provide a cellular and acellular (extracellular matrix production) supporting scaffold in various tissues. Following transplantation into the injured spinal cord, fibroblasts replace cystic lesion defects, however, fibroblasts alone are not sufficient to provide a cellular guiding path for injured CNS axon pathways such as the corticospinal tract (CST) [16-18].

We therefore aimed to investigate if the combination of NPC and fibroblasts can retain NPC in the lesion site and augment axonal growth. Results of the present study indicate that NPC grafted together with primary fibroblasts to the injured spinal cord provide a cellular guiding substrate across the lesion cavity and enhance the growth of lesioned corticospinal axons.

2. MATERIALS AND METHODS

2.1 Animals

Adult female Fischer 344 rats (160-180 g) were used for the isolation of NPC and fibroblasts. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. All efforts were made to minimize the number of animals used and their suffering. Animals had *ad libidum* access to food and water throughout the study. All surgical procedures were performed under anesthesia with a combination of ketamine (62.5 mg/kg; WDT, Garbsen, Germany), xylazine (3.175 mg/kg; WDT, Garbsen, Germany) and acepromazine (0.625 mg/kg, Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution.

2.2 Preparation of Adult NPC and fibroblasts

Rats were deeply anesthetized as described above and killed by decapitation. The cervical enlargement (spinal cord level C3 through T1) was dissected and the dura removed. The tissue was minced, washed in sterile Dulbecco's phosphate buffered saline/D-glucose (4.5g/l; PAA Laboratories, Linz, Austria) and digested in a solution of papain (0.01%; Worthington

Biochemicals, Lakewood, USA), neutral protease (0.1%; Roche, Mannheim, Germany), DNase I (0.01%; Worthington Biochemicals) and 12.4mM $MgSO_4$, dissolved in Hank's balanced salt solution (HBSS; PAA Laboratories, Linz, Austria) for 30 min at 37°C. The tissue was centrifuged at 120 x g for 5 min at 4°C and washed three times in DMEM-HAMS F12 (Pan Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS; Pan Biotech, Aidenbach, Germany). The cells were transferred to culture dishes containing serum-free growth medium, consisting of Neurobasal medium with B27 supplement (Gibco, Karlsruhe, Germany) and 20 ng/ml recombinant human FGF-2 (R&D System, Wiesbaden, Germany). Cells were grown first as neurospheres for 2-3 passages in uncoated culture flasks. The medium was replaced by centrifuging the medium containing neurospheres at 120 x g for 5 min at 4°C, removing the supernatant and resuspending the cells in fresh growth medium. For passaging of neurospheres, the medium containing the neurospheres was collected in a 15 ml centrifuge tube and centrifuged at 120 x g for 5 min at 4°C. The pellet was resuspended in 1 ml of Accutase and incubated at 37°C for 10 min. The neurospheres were resuspended in growth medium, triturated and centrifuged at 120 x g for 5 min at 4°C. The resulting single cell suspensions were seeded at a density of 3000 cells/cm^2 in fresh growth medium. Cells were passaged every second week. For the consecutive 2-3 passages, NPC were grown as monolayers in culture flasks coated with poly-l-ornithine (20µg/cm^2; Sigma) and laminin (0.4µg/cm^2; Sigma). The cell culture medium was changed twice per week. Monolayer cultures were detached by incubation with 40 ml/cm^2 Acccutase (Innovative Cell Tech, San Diego, USA) for 5 min at 37°C. Finally, the cells were centrifuged at 120 x g for 5 min at 4°C and resuspended in fresh growth medium.

For the identification of NPC co-cultured with primary fibroblasts *in vitro* (see below), NPC were genetically modified to express the reporter gene green-fluorescent protein (GFP) using a retroviral vector as previously described [14].

Primary cultures of adult Fischer 344 fibroblasts were generated from skin biopsies and cultivated under standard culture conditions as previously described [5].

2.3 Preparation of adult NPC for transplantation

Adult NPC were incubated with 1 µM bromodeoxyuridine (BrdU; Sigma) in growth medium for 48 h just before transplantation. A sample of single cell suspensions was stained with Trypan Blue (Sigma) and counted using a Neubauer hemocytometer. The remaining single cell suspension was washed twice and resuspended in PBS to yield a final concentration of 0.8 x 10^5 cells/µl for co-transplantation of NPC and fibroblasts (NPC-FF). Fibroblasts were trypsinized and counted in a Neubauer hemocytometer. Cells were washed twice and resuspended in PBS to yield the final concentration of 0.2 x 10^5 cells/µl. Fibroblast and NPC suspensions were mixed before transplantation. For pure

fibroblast grafts (FF), fibroblasts were re - suspended at a concentration of 0.4 x 10^5 cells/µl. Due to the larger cell size of fibro- blasts this suspension was approximately equally dense as the mixed cell grafts.

2.4 In vitro co-cultures of adult NPC and fibroblasts

To study the adhesion behavior of adult NPC in the presence of fibroblasts, GFP expressing NPC were either kept as neu - rospheres in T25 flasks in proliferation medium either without (pure NPC condi - tion) or with primary fibroblasts (NPC-FF condition). Daily for 7 days after the start of co-culturing, the adhesion behavior of NPC was monitored using an inverted phase contrast and fluorescence micro - scope (Olympus).

To analyze the *in vitro* differentiation of NPC, neurospheres of established NPC cultures were dissociated using Accutase (Innovative Cell Tech, San Diego, USA) and seeded on poly-l-ornithine/laminin (P-Orn/Lam, Sigma) coated cover slips either alone (NPC proliferation condition) or together with primary rat fibroblasts at a ratio of 1:2 (NPC-FF proliferation con - dition) for 4 days in serum-free medium (Neurobasal medium with B27 supple - ment and FGF-2 as described above). In parallel, cultures of NPC alone (NPC dif - ferentiation condition) were incubated in medium, consisting of Neurobasal medi - um, B27 supplement and 1% fetal calf se rum (Pan Biotech, Aidenbach, Germany). Cells were subsequently fixed with 4% paraformaldehyde in PBS and immuno - labeled using the following antibodies: mouse-anti-nestin for progenitor/stem cells (Pharmingen, Heidelberg, Germany; at 1/1000), mouse-anti-GFAP for astro - glia (Chemicon, Hofheim, Germany; at 1/600), mouse-anti-A2B5 for glial precur - sor cells (Chemicon, Hofheim, Germany; at 1/100), mouse-anti-Gal-C for oligoden- droglia (Chemicon, Hofheim, Germany; at 1/500) and mouse-anti-beta-III-tubulin for neurons (clone TUJ1; Babco, Richmond, USA; at 1/500).

Cells were washed 3 times with tris- buffered saline (TBS) after fixation, blocked with TBS containing 3% donkey serum/0.1% Triton-X (Sigma) and incu - bated overnight with the primary antibody in TBS + 3% donkey serum + 0.1% Tri - ton-X at 4°C. The following day, cells were washed with TBS and incubated with rho- damine-X linked secondary donkey-anti- mouse antibodies (Jackson, Hamburg, Germany; at 1/1000) in TBS + 3% don - key serum + 0.1% Triton-X for 2h. Finally, nuclei were counterstained with Hoechst 33342 (2 µg/ml in TBS; Sigma). The cover- slips were mounted onto glass slides us - ing Prolong-Antifade (Molecular Probes, Leiden, Netherlands). For the immunocy - tochemical analysis, 8 bit monochrome pictures were taken at 20x magnification on a fluorescent microscope (Leica DMR) equipped with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Sterling Heights, USA). The number of specifi - cally immunolabeled cells and the total cell number (number of Hoechst counter - stained nuclei) were determined in 5 ran - dom fields for each differentiation marker to determine the percentage of cells ex -

pressing the respective differentiation marker. Three independently performed immunocytochemical stains were analyzed under expansion and differentiation conditions.

2.5 Surgical procedures

Spinal cord lesions, cell transplantation and anterograde labeling of the CST were performed as previously described [14, 19, 20]. Cells were grafted immediately post-injury to maximize the potential growth responses of corticospinal axons and to ensure that the injured tips of corticospinal axons are in close proximity to the graft site. Briefly, the dorsal columns containing the dorsal CST were transected at cervical level C3 using a tungsten wire knife (David Kopf Instruments Tujuna, USA) leaving the surrounding dura intact. A total volume of 3 µl of a cell suspension containing either 2.4 x 10^5 NPC (prelabeled with BrdU) combined with 0.6 x 10^5 fibroblasts (**NPC-FF**; n=8) or 1.2 x 10^5 fibroblasts only (**FF**; n=8) was injected directly into the lesion site through a pulled glass micropipette (100 µm internal diameter) using a Picospritzer II (General Valve, Fairfield, USA). Animals receiving spinal cord lesions without cell transplantation (**LESION**; n=6) served as controls.

For anterograde tracing of the CST projections, 300 nl of a 10% solution of biotinylated dextran-amine (BDA; 10.000 MW, Molecular Probes, Leiden, Netherlands) was injected through pulled glass micropipettes (40 µm internal diameter) into each of 18 sites per hemisphere spanning the rostrocaudal extent of the rat forelimb and hindlimb sensorimotor cortex using a PicoSpritzer II [19] 1 week post lesioning/grafting.

2.6 Morphological analysis

At 3 weeks post lesioning/grafting, animals were transcardially perfused with a 0.9% saline solution in 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Sagittal 35 µm cryostat sections were processed for visualization of the BDA labeled CST and for immunohistochemistry. Every seventh section was mounted for Nissl staining to determine the lesion size.

Double/triple labeling immunofluorescence techniques were performed with free floating sections to assess cell survival, *in vivo* differentiation pattern, and interaction of grafted adult NPC with injured axons. The following primary antibodies were used: rabbit-anti-BLBP (for radial glia; generous gift N. Heintz, Rockefeller University, New York, USA; at 1/1000), mouse-anti-GFAP (for astrocytes; DAKO, Glostrup, Denmark; at 1/200), mouse-anti-APC (for oligodendrocytes, Oncogene, Darmstadt, Germany; at 1/1000), rabbit-anti-200 kDa neurofilament (axonal marker; Boehringer Mannheim, Germany; at 1/250). Immunolabeling was visualized using fluorescein linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000). BrdU-prelabeled cells were identified with a rat-anti-BrdU antibody (Harlan SeraLab, Loughborough, UK; at 1/500) visualized using rhodamine X- linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000).

Sections were rinsed in 0.1 M Tris buffered saline (TBS), incubated in TBS + 3% donkey serum + 0.1% Triton-X for 1h, then transferred into the first primary antibody and incubated overnight at 4°C on a rotating platform. For visualization of BrdU-prelabeled adult NPC the staining protocol was modified as follows: after rinsing in TBS, sections were incubated for 1h in 50% formamide/2xSSC (0.3 M NaCl, 0.03M sodium citrate) at 65ºC. Sections were rinsed in 2xSSC, incubated for 30 min in 2 N HCl at 37ºC, and rinsed for 10 min in 0.1 M boric acid pH 8.5. After rinsing in TBS, sections were incubated in TBS + 3% donkey serum + 0.1% Triton-X as described above. The following day, sections were rinsed and incubated with fluorophor (rhodamine-X, fluorescein, Cy5) conjugated secondary donkey antibodies (Jackson, Hamburg, Germany; at 1/1000) for 2.5 h. BDA labeled CST axons were visualized by incubation with Cy5-conjugated streptavidin (Jackson, Hamburg, Germany; at 1/1000). After a final rinsing step in TBS, sections were mounted onto glass slides and coverslipped with ProLong Antifade Kit (Molecular Probes, Leiden, Netherlands).

For brightfield immunohistochemical analysis of axon regeneration rabbit-anti-200-kDa neurofilament (Boehringer Mannheim, Germany; at 1/250) was used as axonal marker.

Brightfield immunohistochemical analysis was performed by incubating free-floating sections for 24 h in primary antibody solution in TBS containing 5% blocking serum and 0.25% Triton X-100; incubation for 1 h with biotinylated donkey IgG (Dianova, Germany; at 1/1000) in TBS containing 5% blocking serum; 1 h incubation with avidin biotinylated peroxidase complex (Vector Elite kit; Wertheim, Germany; at 1/1000) with TBS containing 5% blocking serum; development for 3–15 min in a 0.05% solution of 3,38-diaminobenzidine (DAB), 0.01% H_2O_2, and 0.04% nickel chloride in TBS. For brightfield microscopic immunohistochemical analysis of BDA labeled CST axons, sections were incubated overnight with avidin biotinylated peroxidase complex (Vector Elite Kit, Vector Laboratories, Wertheim, Germany; at 1/1000) with TBS, followed by treatment for 5 min with a 0.05% solution of DAB, 0.01% H_2O_2, and 0.04% nickel chloride in TBS (brown-black reaction product). Tissue sections were mounted onto gelatin-coated glass slides, air dried, dehydrated and coverslipped with Neo Mount (Merck).

Brightfield imaging was conducted on a Leica DMR microscope with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Michigan, USA). Confocal immunofluorescence microscopy was performed using a Leica TCS-NT system.

2.7 Quantification of NPC differentiation in vivo

The *in vivo* differentiation pattern of NPC was analyzed by confocal fluorescence microscopy (Leica TCS-NT). Co-localization of BrdU labeled NPC with differentiation markers was analyzed using 15-20 optical sections along the z-axis of 35 µm thick sections at 400x magnification.

Differentiation markers were considered co-localized, if they surrounded the nuclear BrdU labeling through subsequent optical sections in the z-axis. The *in vivo* differentiation pattern was quantified in one section for each differentiation marker by analyzing 2 scans within the graft at 400x magnification. The total number of BrdU positive cells within each scan was counted and correlated to the number of BrdU positive cells co-localizing with the respective differentiation marker (GFAP, BLBP, APC).

2.8 Quantification of axonal regeneration

For quantification of axon (neurofilament labeling) and CST axon profiles (BDA labeling) within the graft, every eighth section out of all sagittal sections per animal was processed for the visualization of BDA or neurofilament, respectively. Out of these sections, the section containing the center of the graft was selected for quantification of neurofilament labeling, the section covering the main portion of the dorsal CST was chosen for quantification of CST regeneration. Digital brightfield images of neurofilament-DAB or BDA-DAB visualized sections were captured using a Spot CCD camera, model 2.2.1 (Diagnostic Instruments, Michigan, USA) attached to a Leica DMR microscope at 200x magnification. For the quantification of axon profiles, neurofilament-DAB or BDA-DAB positive structures were highlighted by marking them in NIH Image 1.62 with a digital pen, while going through the z-axis of the section, to detect complete labeling of neurofilament/BDA positive axons throughout the depth of the section. The number of pixels representing the marked lines was assessed using NIH Image 1.62 software. To determine the maximum distance of CST axon regeneration into the graft (axon length), the distance between the graft host interface and the longest axon ending was measured using NIH Image 1.62. The graft host interface was determined after inserting the transmitted light condenser, which allows to discriminate between the orderly tissue architecture of the host and the randomly organized cell graft. An observer blinded to group identity rated the degree of axonal regeneration of each animal.

2.9 Statistical analysis

All data are expressed as mean ± SEM. Comparisons between NPC-FF, and FF grafted animals were made by nonparametric Mann-Whitney U tests and $P<0.05$ was considered significant.

3. RESULTS

3.1 Adhesion and differentiation pattern of adult NPC co-cultured with fibroblasts.

Previously, we have shown that the transplantation of NPC suspensions is not sufficient to replace the lesion cavity, which forms after spinal cord injury [14]. To determine if fibroblasts might be able to provide a supporting scaffold for NPC in the injured spinal cord, we first investigated the influences of primary fibroblasts on NPC adhesion and differentiation *in vitro*. Co-cultures of adult GFP expressing

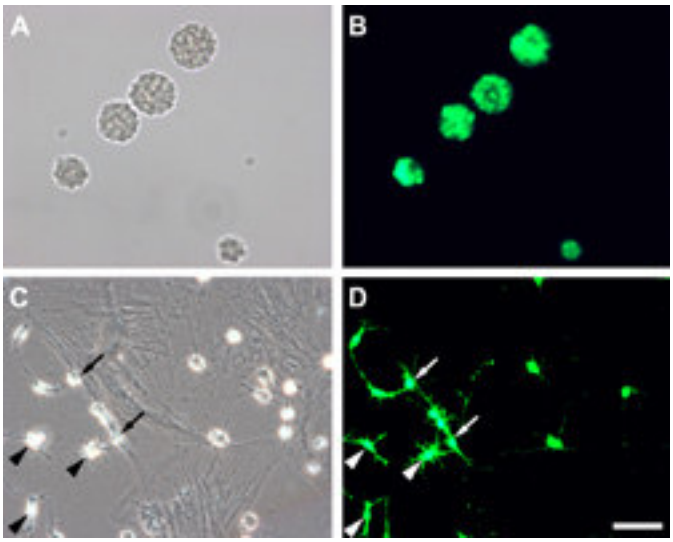

Figure 1: *Adherence of NPC co-cultured with primary fibroblasts: (A) Adult NPC spontaneously form free-floating neurospheres in uncoated culture flasks. Cells do not adhere to the plastic surface. (B) View of the same field as (A) under fluorescent illumination demonstrates GFP expression by NPC. (C) Co-cultivation of NPC with fibroblasts leads to the adherence of NPC to the plastic surface already after 24 hours either independently of surrounding fibroblasts (arrowheads) or directly attached to fibroblasts (arrows). (D) Expression of GFP (arrowheads and arrows corresponding to C) identifies the adhering cells as NPC. (A, C) phase-contrast microscopy, B, D fluorescence microscopy. Scale bar 64 µm*

NPC and skin-derived fibroblasts were prepared on uncoated standard plastic culture flasks and compared to pure NPC cultures. Consistent with previous reports, adult NPC formed free floating cell con - glomerates (neurospheres), which did not attach to the surface of the plastic culture dish (Fig. 1A,B) [14]. In contrast, NPC co-cultured with primary fibroblasts adhered to the uncoated plastic surface within 24 hours (Fig. 1C,D). NPC survived in the presence of fibroblasts, were frequently in direct contact with fibroblasts and formed processes along the fibroblast surface. Although NPC/fibroblast co-cultures were maintained in serum-free FGF-2-contain - ing proliferation medium, NPC differenti - ated into GFAP (31.4% ± 2.7) or Gal-C (5.9% ± 0.5) expressing glial cells with the respective astroglial and oligodendroglial morphology, and beta-III-tubulin (48.9% ± 1.7) expressing neurons (Fig. 2C-E,H). The finding that the sum of individual differentiation markers exceeds 100% is likely due to an overlap between GFAP and A2B5 in astroglial/glial precursor cells as previously described [21]. In contrast, adult NPC maintained as pure NPC cultures displayed antigenicity and morphology of stem/progenitor cells (nestin; 19.2% ± 0.9), glial precursor cells (A2B5; 41.8% ± 1.2) and early neurons (beta-III-tubulin; 22.2% ± 1.8) only (Fig. 2A,B,F). After in - duction of differentiation in pure NPC cultures by adding FCS instead of FGF-2 for 4 days (Fig. 2G), nestin expressing cells

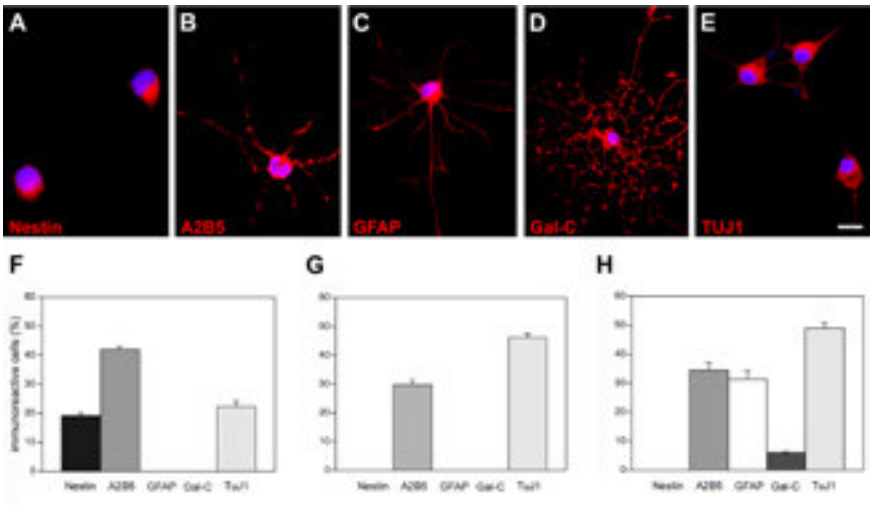

Figure 2: *Differentiation of NPC co-cultivated with fibroblasts: Morphology and immunocytochemical labeling of adult NPC in (A, B) pure NPC cultures and (C-E) NPC/FF co-culture conditions in FGF-2-containing proliferation medium. Cells display immunoreactivity for (A) the stem/progenitor marker nestin, (B) the glial precursor marker A2B5, (C) the astroglial marker GFAP, (D) the oligodendroglial marker Gal-C, and (E) the early neuronal marker beta-III-tubulin (clone TUJ1). All differentiation markers are shown in red, Hoechst 33342 nuclear counterstaining is shown in blue. (F-H) Quantification of the differentiation pattern of adult NPC in vitro 4 days after plating on P-Orn/Lam coated plates: (F) pure NPC cultures in FGF-2 containing proliferation medium, (G) pure NPC cultures in FCS containing differentiation medium and (H) FF-NPC co-cultures in FGF-2 containing proliferation medium. Scale bar A 5.7 µm, B 6.1 µm, C 8.2 µm, D 10.9 µm, E 8.2 µm.*

disappeared completely and glial precursor cells expressing A2B5 were reduced (29.5% ± 1.8). The number of beta-III-tubulin expressing cells increased to 45.9% (± 1.4). In contrast to previous studies using comparable cell culture conditions, GFAP and Gal-C expressing cells were still absent in differentiation conditions [13, 14]. However, in these studies a longer incubation period in differentiation medium (7 days versus 4 days in the present differentiation assay) was chosen. It has been suggested that the neuronal marker beta-III-tubulin is expressed in astroglial cells [22]. However, in a previous experiment we did not detect any co-localization in adult NPC *in vitro* in a double immunofluorescent analysis with beta-III-tubulin and GFAP (unpublished observation). Taken together, fibroblasts and extracellular matrix molecules secreted by fibroblasts provided a supporting scaffold for NPC to adhere to in culture. The presence of fibroblasts shifted the differentiation pattern of FGF-2 responsive adult NPC towards glial phenotypes.

3.2 Cyst replacement, survival and cell differentiation

Pure NPC (4.8-5.4 x 10^5 cells per graft) grafted immediately after a cervical dorsal column transection [14] or delayed 1-2 weeks later (unpublished observation) were not sufficient to replace or even

reduce the cystic lesion defect. To determine whether fibroblasts are able to maintain NPC within the lesion site, NPC and fibroblasts were co-grafted as mixed cell suspension (**NPC-FF**) directly into the lesion site following an acute cervical wire knife lesion in adult rats. Animals receiving lesions without grafting (**LESION**) or pure fibroblast grafts (**FF**) served as controls. Three weeks post-lesioning, animals without transplants developed lesion cavities in the dorsal half of the spinal cord. In contrast, lesion cavities were completely replaced in all animals with NPC-FF and FF grafts (Fig. 3). Grafts integrated well into the host parenchyma. There were no signs of uncontrolled graft proliferation or tumor formation in NPC-FF and FF grafted animals. Three weeks after NPC-FF transplantation, adult NPC survived throughout the graft as indicated by the presence of BrdU labeled NPC within the graft (Fig. 4A,C-E), in contrast to the absence of BrdU immunoreactivity in FF grafts (controls) (Fig. 4B). NPC were found primarily within the graft, only few BrdU labeled NPC were identifiable in the host parenchyma adjacent to the graft (Fig. 4A). NPC were not evenly distributed in all NPC-FF co-grafts. In some animals, NPC were found in clusters in the center of the graft (Fig.4A), in others they were found predominantly along the graft-host border, but still within the graft (Fig.4C-E).

BrdU labeled NPC co-grafted with fibroblasts displayed antigenic properties of astroglial (GFAP positive; 43.5% ± 2.3), radial glial (BLBP positive; 44.2% ± 3.5)

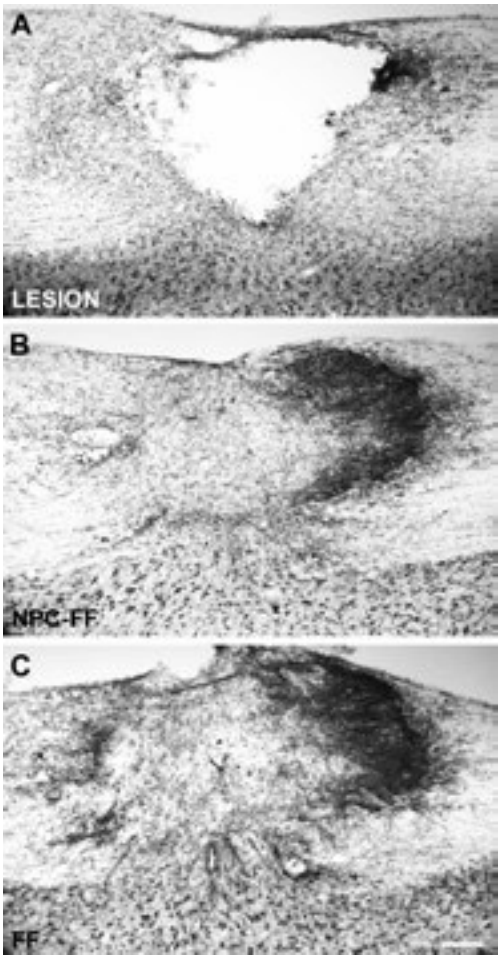

Figure 3: *Cystic lesion replacement: (A) Three weeks following a cervical wire knife dorsal column transection a typical triangular shaped cystic lesion defect develops in animals without transplantation. (B) NPC-FF co-grafts completely replace the cystic lesion cavity and integrate into the host spinal cord. (C) FF grafts also reduce the lesion defect completely. A-C sagittal Nissl stained sections; rostral left, dorsal top; scale bar A-C 196 µm.*

and oligodendroglial cells (APC positive, GFAP negative; 17.8% ± 3.1) (Fig. 5). The percentage of the individual differentiation markers adds up to more than 100% (exactly 105.5%), which is likely due to an overlap between GFAP and BLBP in astroglial cells as previously described [23].

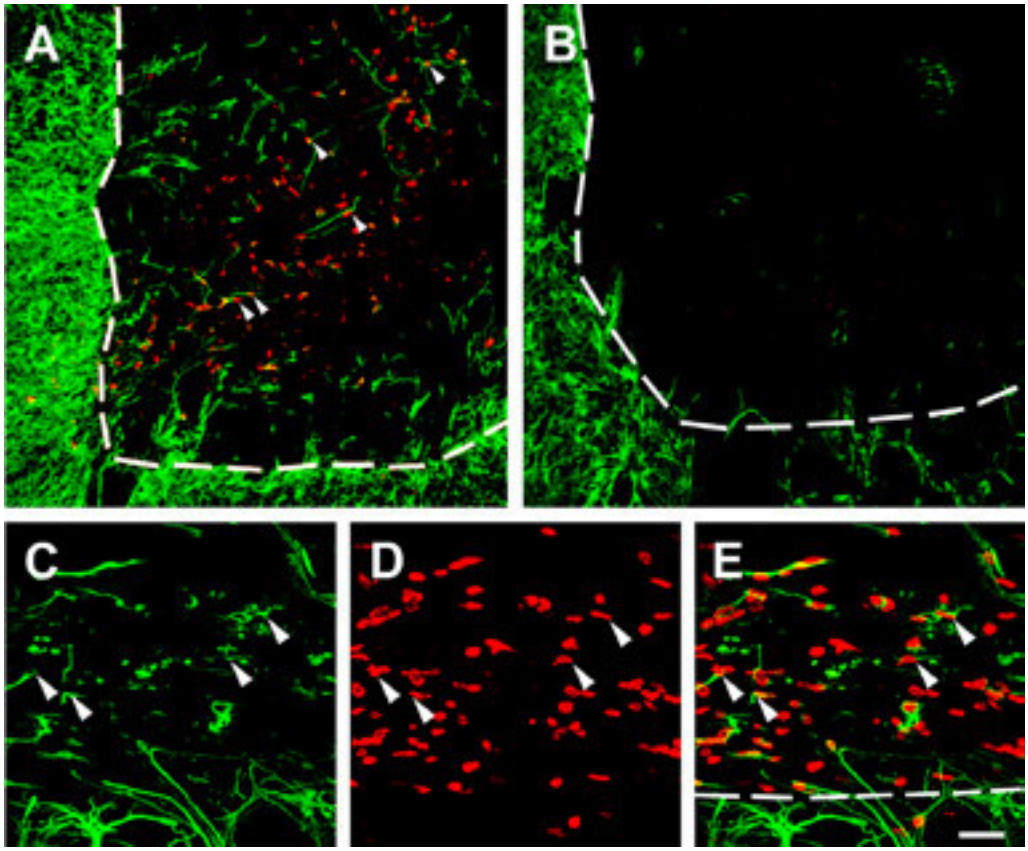

Figure 4: *NPC survival and distribution: (A) Immunohistochemical detection of BrdU prelabeled NPC (rhodamine labeled; red) throughout the graft illustrates that NPC co-grafted together with primary fibroblasts into the acutely injured spinal cord survive. A significant proportion of grafted NPC can be co-localized (arrowheads) with GFAP antigenicity (fluorescein labeled; green) indicating glial differentiation. (B) As control, BrdU is not detectable within FF grafts, GFAP expression is restricted to the surrounding host spinal cord. GFAP expressing processes reach into the fibroblast graft only for a very limited distance. (C-E) The graft-host interface is shown at higher magnification in another NPC-FF grafted animal. (C) GFAP immunolabeled cells, (D) BrdU labeled grafted NPC and (E) merged micrograph of (C) and (D). Arrowheads highlight BrdU/GFAP co-localizing grafted NPC. Orientation in all micrographs: dorsal is at the top, rostral to the left. Dashed lines delineate the graft/host transition. Confocal fluorescence micrograph; scale bar A, B 81,5 µm, C-E 49 µm.*

The majority of GFAP positive NPC displayed bipolar processes rather than multipolar/stellate processes typically found in mature or reactive astrocytes (Fig.5A-C, 6D-G). Consistent with previous studies [13, 14], none of the BrdU prelabeled cells differentiated into a neuronal phenotype as indicated by the lack of beta-III-tubulin – BrdU colocalization (data not shown).

3.3 Axonal regeneration

The maintenance of adult NPC within the lesion cavity of NPC-FF co-transplant recipients allowed us for the first time to investigate the capacity of NPC to promote axon regeneration into the graft. Axonal

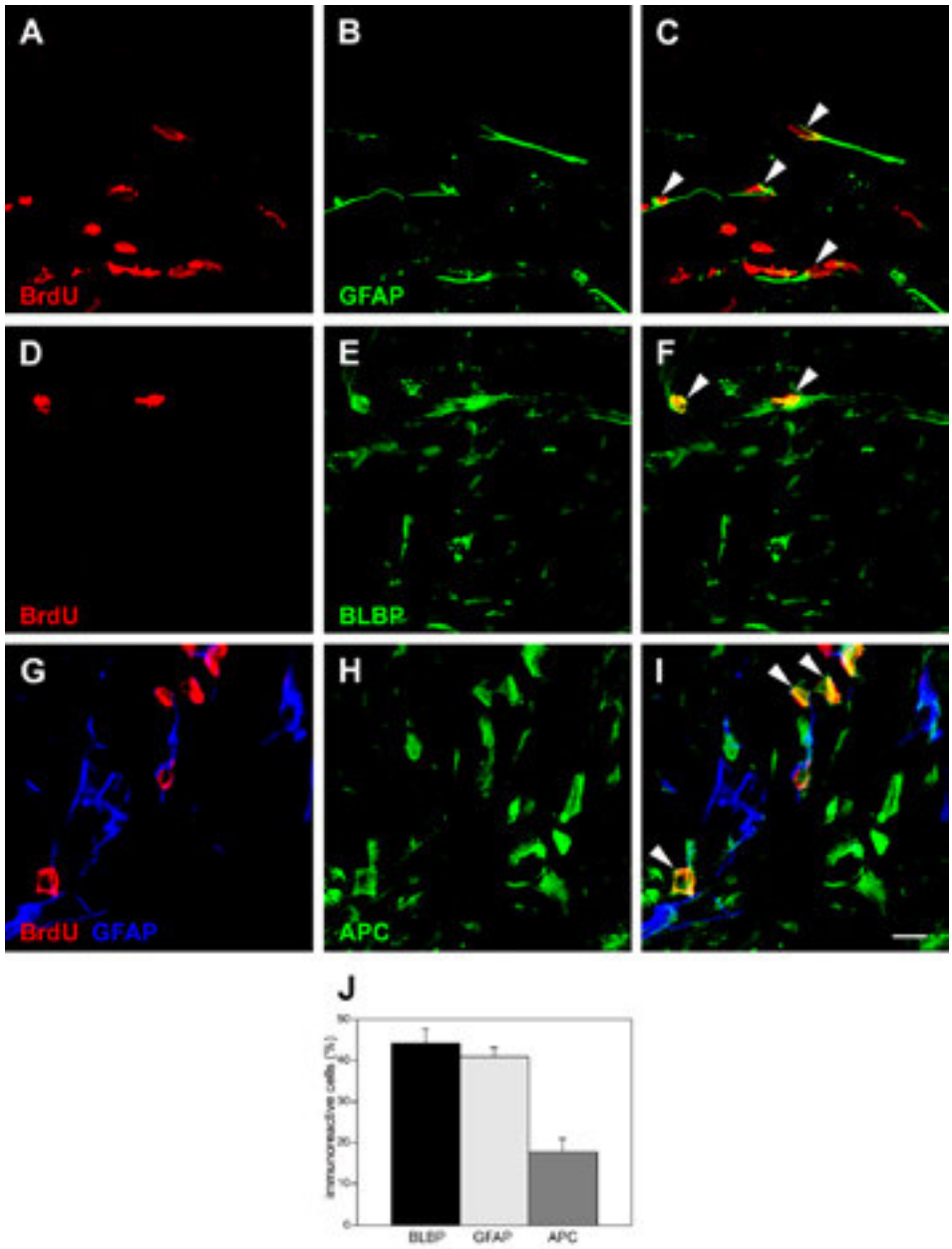

Figure 5: *Confocal analysis of NPC differentiation in NPC-FF co-grafts: (A-C) BrdU prelabeled NPC co-localize with GFAP immunoreactivity (arrowheads). GFAP expressing cells frequently display bipolar elongated processes reminiscent of immature or radial glial cells in the developing CNS. (D-F) NPC are also co-localized with BLBP (arrowheads), which is typically expressed in radial glial cells. (G-I) NPC immunoreactive for APC and at the same time negative for GFAP (Cy5 labeled; blue) indicate oligodendroglial differentiation (arrowheads). The BrdU labeled NPC, co-localized with APC in the center of the field, is also co-localized with GFAP suggesting astroglial rather oligodendroglial differentiation. (C, F, I) Merged images of the rhodamine, fluorescein and Cy5 channels. (J) Quantification of BrdU prelabeled NPC co-localized with BLBP, GFAP or APC. Scale bar A-C, 27.4 µm, D-F 24.4 µm, G-I 15.9 µm.*

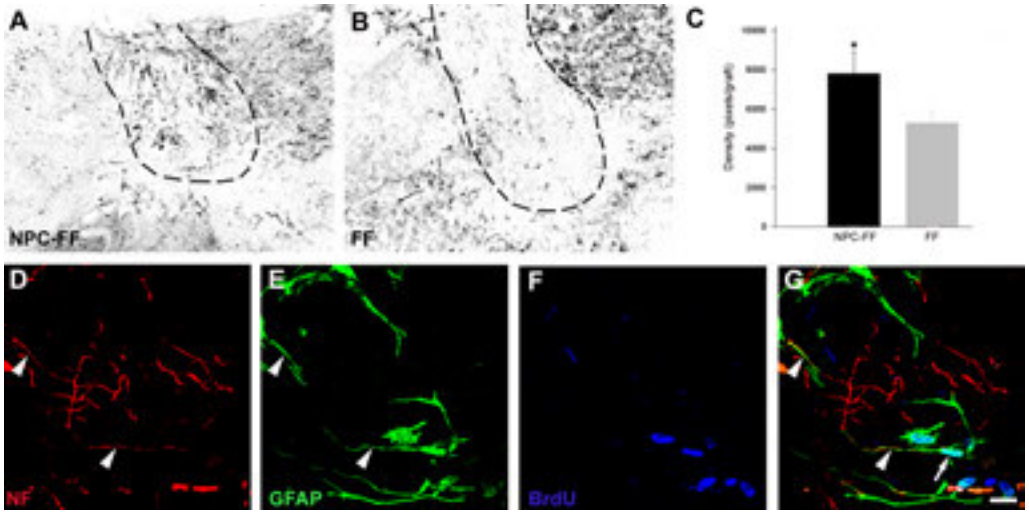

Figure 6: *Axonal growth into fibroblast (FF) and NPC/fibroblast grafts (NPC-FF): Representative micrographs of neurofilament expressing axonal profiles within (A) NPC-FF and (B) FF grafts. Dashed lines delineate the graft/host interface. (C) Quantification of neurofilament labeling reveals significantly more axonal growth into co-grafts ($p<0.05$). Error bars represent SEM. (D) Neurofilament (NF) expressing axons within the graft are longitudinally oriented along (E) GFAP labeled cells (highlighted by arrowheads in D, E and G), which are colocalized with (F) BrdU prelabeled NPC (highlighted by arrow in G). (G) Merged image of (D-G). (A, B) Immunohistochemical brightfield images, (D-G) confocal immunofluorescence images. Orientation in all images: rostral to the left, dorsal at the top. Scale bar A, B 85.7 µm, D-G 14.8 µm.*

growth into NPC-FF co-grafts was compared to FF grafts by quantifying the number of neurofilament labeled axonal profiles. Axonal growth was observed into both, FF grafts and NPC-FF co-grafts. However, the number of axonal profiles was significantly increased by 47 % into NPC-FF co-grafts (7809 ± 1120 pixels/graft) versus fibroblast grafts (5299 ± 618 pixels/graft; $p < 0.05$) (Fig. 6A-C). Neurofilament labeled axons within NPC-FF co-grafts were frequently oriented longitudinally along GFAP labeled cellular processes (Fig. 6D-G), which co-localized with BrdU labeled nuclei. GFAP expressing cells in the graft typically displayed a bipolar, immature morphology. The cervical wire knife lesion completely disrupts the main portion of the CST descending in the dorsal columns of the spinal cord. Therefore, the presence of anterogradely BDA labeled axonal profiles traversing from the main dorsal pathway into the graft indicates regrowth of transected CST axons rather than sprouting responses of uninjured ventral corticospinal axons. In analogy to neurofilament labeled axons, significantly more CST axons regenerated into NPC-FF co-grafts (2774 pixels ± 384) in comparison to FF grafts (1027 pixels ± 356; $p<0.05$) (Fig. 7A-C). In addition, the distance of CST axon regrowth into the graft measuring the longest distance of BDA labeled CST axon endings from the graft-host interface into the graft was significantly enhanced by 69% in NPC-FF co-grafts (316 mm ± 40) versus FF grafts

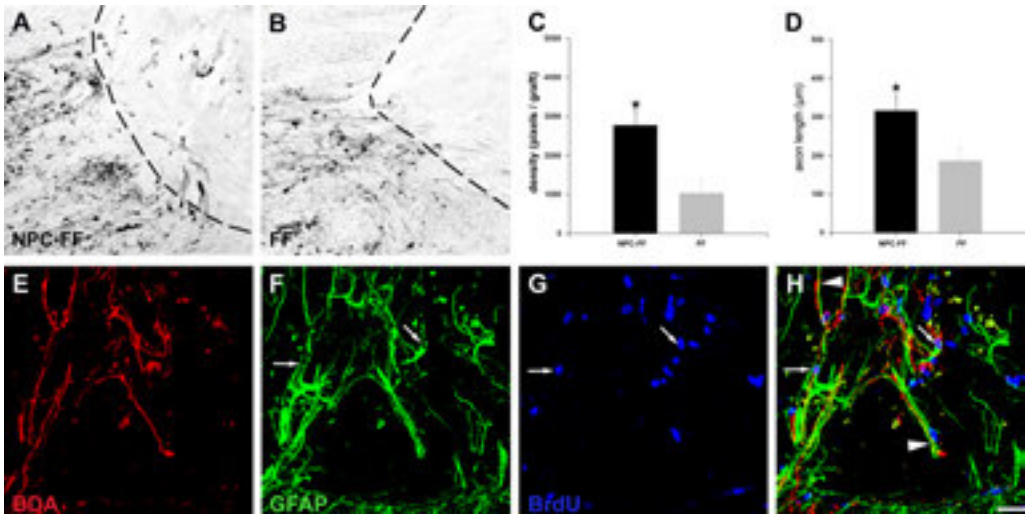

Figure 7: *Corticospinal axon regeneration: Representative micrographs of BDA labeled corticospinal axons within (A) NPC-FF and (B) FF grafts. Dashed lines delineate the graft/host transition. (C) Quantification of BDA labeling reveals significantly more axonal growth into NPC-FF grafts ($p<0.05$). (D) The distance of CST axon growth into the graft is significantly higher in NPC-FF grafts compared to FF grafts ($p<0.05$). Error bars represent SEM. (E) BDA labeled CST axons are longitudinally aligned along (F) GFAP expressing cells, which are colocalized with (G) BrdU prelabeled nuclei of grafted NPC (highlighted by arrows in F- H). (H) Merged images of (E-G). Axon endings with morphological features of growth cones (arrowheads) are oriented along GFAP positive processes, indicating that axons use GFAP expressing cells as a guiding scaffold. (A, B) Immunohistochemical brightfield micrographs, (E-H) confocal immunofluorescence micrograph. Orientation in all images: rostral to the left, dorsal at the top. Scale bar A, B 36.4 µm, E-H 25.5 µm.*

(187 mm ± 35; $p<0.05$) (Fig. 7D). Similar to neurofilament labeled axons, BDA labeled CST axons were aligned along GFAP positive processes in the graft (Fig. 7E-G). BDA labeled CST axon endings dis - played morphological features of growth cones, strongly suggesting an ongoing process of axonal regrowth along cellular processes rather than grafted cells align - ing their processes along axons within the grafts. GFAP positive cells showed a bipolar morphology with long processes and co-localized with BrdU labeled nu - clei, identifying them as NPC derived cells (Fig. 7H). These data indicate that grafted NPC-derived GFAP expressing cells have the capacity to build a physical guiding structure for regenerating CST axons.

4. DISCUSSION

Findings of the present study indicate that grafted adult neural progenitor cell (NPC) derived glial cells enhance regeneration of CNS axons following spinal cord injury. NPC grafts facilitate axonal regeneration by providing a growth permissive guid - ing substrate mimicking axonal outgrowth pattern in the developing CNS.
Studies in lower vertebrates indicate that glial cells play an important role as guidance cues for axon growth and regeneration during development and after injury [24, 25]. The role of direct cellular guid- ance in axonal growth during development

and after injury is much less defined in the mammalian CNS. Axonal pathfinding in the corpus callosum of developing rats has been associated with cellular guidance cues provided by astroglial cells[26, 27]. Time course studies in the postnatal mammalian spinal cord demonstrate that corticospinal axons growing throughout the spinal cord between postnatal day 0 and 10 are in close spatial and temporal association with processes of vimentin and GFAP expressing glial cells[7]. *In vitro* studies have shown that astroglial cells directly guide neurite outgrowth only at a distinct immature developmental stage[28, 29]. There, growth promoting astroglial cells display a bipolar morphology with long processes[28]. This bipolar morphology can be found in grafted GFAP expressing NPC in our study indicating that these cells represent an immature glial cell type rather than mature astrocytes. Therefore, the axonal growth observed in our experiments likely recapitulates cell guided axon outgrowth during development.

Co-cultivation of NPC with fibroblasts significantly increased the percentage of cells differentiating into a glial GFAP expressing phenotype in our *in vitro* experiments. Previous *in vitro* studies also demonstrated that mesenchymal cells such as meningeal cells or corneal fibroblasts promote glial differentiation in fetal cortex derived cell cultures or postnatal cerebellar slice cultures [30, 31]. Interestingly, meningeal cells also induced a bipolar morphology in GFAP expressing cells [31] similar to the glial morphology of NPC co-grafted with fibroblasts. Whether these effects are mediated through direct cell-cell interactions or through soluble factors remains to be determined. Further evidence supporting the concept that fibroblasts might promote a more bipolar glial phenotype resembling immature astroglial cells, is provided by previous observations[14]. After transplantation of pure NPC, isolated and cultivated under the same conditions as in the present study, GFAP immunolabeled cells frequently displayed a mature, stellate appearance.

We observed an increase in axonal sprouting into NPC-FF grafts compared to control fibroblast grafts despite the presence of putative inhibitory extracellular matrix molecules such as chondroitin sulfate proteoglycans, which have been detected within and around fibroblast containing grafts following spinal cord injury [18]. It is possible that NPC influence the local expression or degradation of inhibitory extracellular matrix molecules to an extent that cannot be determined by immunohistochemistry. This notion is supported by recent findings demonstrating that immature glial cells are capable to overcome the growth-inhibitory barrier by digesting specifically the CSPG aggrecan in *in vitro* outgrowth assays [32]. Alternatively, axon growth promoting glial cells could express growth permissive signals, which outweigh inhibitory signals from various extracellular matrix molecules present within the graft [18]. In this context, it is interesting to note that growth permissive signals such as the cell adhesion molecules NCAM and L1 have been identified on growth promoting immature astroglia, however, not on mature astroglia[29].

It becomes evident from this study that any restorative strategy employing neural stem cells for transplantation without a supporting scaffold following spinal cord injury is not sufficient to replace extensive lesion defects developing in the course of a traumatic spinal cord lesion, and therefore cannot provide a bridge for axons to regenerate on. The necessity to administer a supporting scaffold together with neural stem cells has already been demonstrated following transplantation into an ischemia induced CNS lesion [15]. However, in the present study naturally occurring fibroblasts and/or extracellular matrix molecules released by fibroblasts as opposed to previously used artificially manufactured polymers were able to maintain adult NPC within the cystic lesion defect. Some extracellular matrix molecules secreted by fibroblasts such as laminin or fibronectin have been found to enhance cell motility, and thereby might accelerate the migration of adult NPC away from the graft into the surrounding host spinal cord. However, other extracellular matrix molecules such as CSPG, which are expressed in fibroblast containing grafts [18], actually inhibit NPC migration [33]. Thus, CSPG might be an important component to prevent significant migration of NPC away from the lesion site and to maintain NPC within the lesion cavity.

In terms of a putative clinical application, the question remains whether adult NPC are a cell population superior to fibroblasts alone or to fetal spinal cord tissue containing immature neural cells, which are already placed in a predefined tissue architecture before grafting. Both strategies have been shown to replace cystic lesion defects and to promote partial functional recovery following acute and chronic spinal cord injury [17, 34-40]. In particular, fibroblasts genetically modified to secrete regrowth promoting neurotrophic factors such as neurotrophin-3 (NT-3) or brain derived neurotrophic factor (BDNF) elicit regeneration of supraspinal projections into the graft, however, axons are unable to reenter the host spinal cord [35, 39]. Thus, fibroblasts represent a powerful cellular vehicle to locally deliver growth promoting molecules, however, they do not have the capacity to serve as a “bridge” reconnecting injured rostral axons with targets located distally or vice versa. Fetal spinal cord tissue has been demonstrated to elicit functional recovery, but the exact structural mechanisms remain to be determined [34, 38, 41]. Ethical concerns and the limited availability of fetal transplants further restrict their clinical applicability. Adult NPC represent a highly promising source for regenerative cell based-strategies following spinal cord injury. However, at the time point investigated axon growth is still limited. Axons do not regenerate beyond a limited distance within the transplant and do not re-enter the caudal host spinal cord. Future studies need to investigate axonal regeneration over longer periods of time. The exact characteristics of growth promoting glial cells need to be specifically identified and these cell populations need to be enriched to augment structural and potentially functional recovery. Increasing the ratio of NPC to fibroblasts, overexpressing neurotrophic

factors such as NT-3[17], and stimulating the neuronal cell body with cyclic nucleotides [42] might be other means to enhance the regenerative capacity of axons into and beyond NPC containing grafts. Studies in the chronically injured spinal cord are needed to determine whether NPC survival/differentiation and corticospinal growth responses are similar in chronic spinal cord injuries compared to acute injuries investigated in the present study.

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