Cellular therapy after spinal cord injury using neural progenitor cells

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Chapter 2

Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways

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ABSTRACT

The main rationale for cell-based therapies following spinal cord injury are the 1) replacement of degenerated spinal cord parenchyma by an axon growth supporting scaffold, 2) remyelination of regenerating axons and 3) local delivery of growth promoting molecules. A potential source to meet these requirements are adult neural progenitor cells, which were examined in the present study. Fibroblast growth factor 2 responsive adult spinal cord derived syngenic neural progenitor cells were either genetically modified *in vitro* to express green fluorescent protein (GFP) using retroviral vectors or prelabeled with bromodeoxyuridine (BrdU). Neural progenitor cells revealed antigenic properties of neurons and glial cells *in vitro* confirming their multipotency. This differentiation pattern was unaffected by retroviral transduction. GFP expressing or BrdU prelabeled neural progenitor cells were grafted as neurospheres directly into the acutely injured rat cervical spinal cord. Animals with lesions only served as controls. Three weeks postoperatively, grafted neural progenitor cells integrated along axonal profiles surrounding the lesion site. In contrast to observations in culture, grafted neural progenitor cells differentiated only into astro- and oligodendroglial lineages supporting the notion that the adult spinal cord provides molecular cues for glial, but not for neuronal differentiation. This study demonstrates that adult neural progenitor cells will survive after transplantation into the acutely injured spinal cord. The observed oligodendroglial and astroglial differentiation, and integration along axonal pathways represent important prerequisites for potential remyelination and support of axonal regrowth.

1. INTRODUCTION

The traumatic lesion of the mammalian spinal cord is followed by the degeneration of spinal cord specific cells such as astroglia, oligodendroglia and neurons in and around the lesion site. Ultimately a cystic lesion defect will persist, which, besides the lack of growth promoting molecules and the upregulation of growth inhibitory components [1-4], represents a key factor impeding the regeneration of injured axons. The extent of intrinsic cell renewal alone [5], even after application of mitogenic agents such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) [6, 7], is not sufficient to allow substantial recovery following spinal cord injury [8]. Therefore, exogenous cell replacement strategies have to be considered.

Ideally, cellular candidates for transplantation should be able to replace the function of astrocytes, which build the cellular scaffold of the spinal cord parenchyma and may provide guidance cues for regenerating axons, and oligodendrocytes, which myelinate axons, thus allowing proper nerve conduction. The replacement of neurons is negligible in spinal cord injury, since only neurons at the injured segmental level are lost and therefore contribute only minimally to the

functional deficits observed after spinal cord injury. The use of cellular grafts as vehicles to locally deliver growth promoting molecules is desirable. Various cell types such as fibroblasts, Schwann cells and olfactory ensheathing cells have been analyzed for their regenerative capacity after transplantation into the injured spinal cord [9-14]. Cell based therapies were able to substitute for the loss of glial cells to some degree and to mediate the application of growth promoting factors, however, structural and functional recovery was moderate at best.

Organotypic cell replacement can be achieved with neural progenitor cells (NPC). NPC from embryonic as well as adult central nervous system (CNS) tissue have the capacity for self-renewal and multipotency [15, 16]. After delayed transplantation of embryonic derived NPC into the injured rat spinal cord differentiation into glial and neuronal lineages as well as modest functional improvement have been reported [17-19]. However, ethical concerns and the limited availability restrict the large-scale use of embryonic derived NPC. To prevent rejection of allogenic embryonic cells after transplantation, suppression of the host immune system is required, which represents another major disadvantage of embryonic derived neural cell grafts. In contrast, the patients' own cells could be utilized to obtain adult NPC, thus avoiding issues regarding chronic immunosuppression and ethical concerns, as they would apply for embryonic cell sources. Transplantation of adult NPC into the intact spinal cord of adult rats revealed that grafted cells survived, migrated over considerable distances within the spinal cord and differentiated into astroglial and oligodendroglial cells [20]. Subventricular zone derived adult NPC survived after delayed transplantation (at least 7 days after the injury) into the spinal cord parenchyma surrounding the spinal cord injury site [21]. Grafted adult NPC differentiated into astroglial lineages; oligodendroglial and neuronal differentiation was not observed in the lesion condition. The aim of the present study was to determine whether adult spinal cord derived NPC will survive transplantation directly into the acutely injured spinal cord lesion site, replace degenerated spinal cord parenchyma and promote regeneration of selectively disrupted corticospinal tract (CST) axons.

2. MATERIALS AND METHODS

2.1 Animal subjects

Adult female Fischer 344 rats weighing 160-180 g were used as donors for the isolation of NPC and for the transplantation experiments. 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.

2.2 Preparation of adult NPC

Rats were deeply anesthetized using a cocktail 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 and killed by decapitation. The region of the complete cervical enlargement (spinal cord level C3 through T1) was dissected out. After removal of the dura, the tissue was minced, washed in sterile Dulbecco's phosphate buffered saline/D-glucose (4.5 g/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 12.4 mM $MgSO_4$, dissolved in Hank's balanced salt solution (HBSS; PAA Laboratories, Linz, Austria) for 30 min at 37°C. The digested 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, which consists of Neurobasal medium with B27 supplement (both Gibco, Karlsruhe, Germany) and 20 ng/ml recombinant human FGF-2 (R&D System, Wiesbaden, Germany). Neurobasal medium with B27 supplement has been shown to substantially increase the proliferation rate of NPC *in vitro* [22] as compared to standard proliferation medium consisting of DMEM/F12 and N2 supplement[23, 24]. Cells were either grown as neurospheres in uncoated cell culture flasks or as adherent monolayers in culture flasks, which were coated as follows: flasks were incubated with 20 µg/cm^2 poly-l-ornithine (Sigma) in distilled H_2O for 2 h at 37°C, rinsed and incubated with 0.4µg/cm2 laminin (Sigma) in PBS for 2 h at 37°C. The cell culture medium was changed twice per week. In neurosphere cultures, the medium was replaced by centrifuging the medium containing neurospheres at 120 x g for 5min at 4°C, removing the supernatant and resuspending the cells in fresh growth medium. Cell cultures were passaged in 2 week intervals. Monolayer cultures were detached by incubation with 40 ml/cm^2 Acccutase (Innovative Cell Tech, San Diego, USA) for 5min at 37°C. Finally, the cells were centrifuged at 120 x g for 5min at 4°C and resuspended in fresh growth medium. Passaging of neurospheres was performed as follows: the medium containing the neurospheres was collected in a 15 ml centrifuge tube and centrifuged at 120 x g for 5min 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. After counting an aliquot of the resulting single cell suspensions in a hemocytometer, 3000 cells/cm^2 were plated in fresh growth medium.

2.3 Retroviral transduction of adult NPC

Adult NPC were genetically modified to express the reporter gene green-fluorescent protein (GFP) as previously described for Schwann cells and fibroblasts [13, 25]. The coding sequence for GFP was cloned into the multiple cloning site of the Moloney leukemia virus-derived retroviral

vector pLXSN (Clontech, Heidelberg, Germany). The 293T cell line based Phoenix amphotrophic producer cells (Gary Nolan, Stanford, USA) were grown in DMEM medium supplemented with 10% FCS and transfected with the retroviral construct. After two days, the virus-containing medium was collected, filtered through a 0.45 µm syringe filter and stored at -80°C. The resulting virus contained the neomycin resistance gene under control of the SV40 promoter and the GFP transgene under control of the constitutively active Moloney murine sarcoma virus-derived 5' LTR promoter sequences.
Adult NPC taken from passage number 6 neurospheres were split 1:3 one day before transduction. The cells were incubated for 8 h on two consecutive days with retrovirus containing growth medium supplemented with 1 µg/ml Polybrene (Sigma). To select for cells, which integrated the retroviral vector, G418 (500 µg/ml; Gibco Karlsruhe, Germany) was added to the growth medium. Successful incorporation of the transgene was confirmed by detection of GFP using an inverted fluorescence microscope (Olympus IX 70).

2.4 In Vitro immunocytochemistry

To obtain monolayer cultures, neurospheres of established cultures were dissociated using Accutase (Innovative Cell Tech, San Diego, USA) and plated on poly-l-ornithine/laminin (P-Orn/Lam, Sigma) coated glass coverslips. To induce differentiation, the cells were incubated for 7 days in medium, in which FGF-2 was replaced by 1% FCS (Pan Biotech, Aidenbach, Germany).
The following antibodies were used to analyze the differentiation pattern of adult NPC *in vitro*: mouse-anti-nestin for progenitor/stem cells (Pharmingen, Heidelberg, Germany; at 1/1000), mouse-anti-GFAP for astroglia (Chemicon, Hofheim, Germany; at 1/600), mouse-anti-GalC for oligodendroglia (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 three times with tris-buffered saline (TBS) after fixation with 4% paraformaldehyde in PBS, blocked with TBS containing 3% donkey serum/0,1% Triton-X (Sigma) and incubated overnight with the primary antibody in TBS + 3% donkey serum + 0.1% Triton-X at 4°C. The following day, cells were washed with TBS and incubated with rhodamine-X linked secondary donkey-anti-mouse antibodies (Jackson, Hamburg, Germany; at 1/1000) in TBS + 3% donkey serum + 0.1% Triton-X for 2h. Finally, nuclei were counterstained with Hoechst 33342 (2 µg/ml in TBS; Sigma). The coverslips were mounted onto glass slides using Prolong-Antifade (Molecular Probes, Leiden, Netherlands). For the immunocytochemical 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). Three independently performed immunocytochemical stains were analyzed in expansion and differentiation conditions. Immunoreactive cells were determined in

Table 1: *Immunohistochemical Markers for Adult NPC Differentiation*

Neural progenitor cells	Nestin (Pharmingen)
Glial precursor cells	NG2 (B. Stallcup)
Radial glia	BLBP (N. Heintz)
Astroglia	GFAP (DAKO)
Oligodendroglia	GalC (Chemicon), APC (Oncogene)*
Neurons	Beta-III-tubulin, (Babco, Promega)

GalC was used for detection of oligodendroglial differentiation in vitro only. In vivo only cells, which were APC positive and at the same time GFAP negative, were classified as oligodendroglia.

5 random fields per immunocytochemical stain in relation to all cells present within the field (= number of Hoechst counter - stained nuclei).

2.5 Preparation of adult NPC for transplantation

Adult NPC, either retrovirally transduced to express the reporter gene GFP as described above (NPC-GFP) or incu - bated 48h before transplantation with a 1µM solution of the proliferation marker bromodeoxyuridine (BrdU; Sigma) in the growth medium (NPC-BrdU), were trans - planted. To control for the stable expres - sion of the GFP reporter gene, a subgroup of adult GFP expressing NPC was prela - beled with BrdU (**NPC-GFP/BrdU**). The respective cells, kept as neurospheres in uncoated cell culture flasks, were cut into fragments (average size 200µm) using a McIlwain Tissue Chopper (Mickle Engi - neering, Gomshall, United Kingdom) [26]. To estimate the number of adult NPC, three samples (100µl each) from uncoated cell culture flasks containing the neurosphere fragments were taken and dissociated with Accutase (Innovative Cell Tech, San Diego, USA). The resulting single cell sus- pension was stained with Trypan Blue (Sigma) and counted using a Neubauer hemocytometer. The remaining neuro - sphere fragments were washed twice and resuspended in PBS to yield the final con- centration of 1.6-1.8 x 10^5 cells/µl ready for transplantation.

2.6 Surgical procedures

Animals were anesthetized using a cock - tail of ketamine, xylazine and aceproma - zine as described above. Spinal cord dor- sal column transections at cervical level C3 and anterograde labeling of the CST were performed as previously described [27, 28].

After a stereotactically guided transection of the dorsal CST with a tungsten wire knife (David Kopf Instruments, Tujuna, USA) at cervical level C3, a total volume of 3 µl cell suspension containing 4.8-5.4 x 10^5 cells (n=8 **NPC-BrdU**; n=8 **NPC- GFP**; n=4 **NPC-GFP/BrdU**) was inject - ed directly into the lesion site through a pulled glass micropipette (200µm internal diameter) using a Picospritzer II (General Valve, Fairfield, USA). Animals receiving spinal cord lesions without cell transplan- tation (n=6) served as controls. Superna - tant from the last washing step after BrdU incubation was injected into 2 animals

(3µl per animal) to control for extracellular BrdU contamination in the cell suspension, which would produce unspecific labeling of proliferating host cells.
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 [27] 1 week post lesioning/grafting.

2.7 Morphological analysis

At 3 weeks post lesioning/grafting, animals were transcardially perfused with a 0.9% saline solution followed by 4% paraformaldehyde in PBS. Sagittal 35µm cryostat sections were processed for visualization of the BDA-labeled CST and for immunohistochemistry. Every seventh section was taken for Nissl staining to determine the cystic lesion size.
Double/triple labeling immunofluorescence techniques were performed to assess cell survival, differentiation pattern and interaction of grafted adult NPC with injured CST 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), rabbit-anti-GFAP (for astrocytes; DAKO, Glostrup, Denmark; at 1/200), mouse-anti-APC (for oligodendrocytes, Oncogene, Darmstadt, Germany; at 1/1000), mouse-anti-beta-III-tubulin (for neurons; Promega, Mannheim, Germany; at 1/100) and rabbit-anti-NG2 (for glial restricted precursor cells; generous gift B. Stallcup, Burnham Institute, La Jolla, USA; at 1/500), all visualized using rhodamine-X linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000). BrdU-prelabeled grafted cells were identified with a rat-anti-BrdU antibody (Harlan SeraLab, Loughborough, UK; at 1/500) visualized using fluorescein linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000). GFP expressing grafted NPC were visualized by using a rabbit-anti-GFP antibody (Molecular Probes, Leiden, Netherlands; at 1/750).
To assess the number of dividing grafted NPC double immunofluorescence labeling was performed with the proliferation marker mouse-anti-Ki-67 (Dianova, Hamburg, Germany; at 1/50).
Sections were rinsed in TBS (0.1M), 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 30min in 2 N HCl at 37ºC, and rinsed for 10min in 0.1 M boric acid pH 8.5. After rinsing in TBS, the protocol continued with the incubation in TBS + 3% donkey serum + 0.1% Triton-X as described above. The following day, sections were rinsed and incubated with fluo-

rescence (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). Immunohistochemical analysis was performed with a confocal fluorescence microscope (Leica TCS-NT). Co-localization of BrdU prelabeled NPC with the individual differentiation marker was determined by analyzing between 15-20 optical sections through the z-axis of a 35 µm thick section. Co-localization was confirmed, once the differentiation marker was spatially associated to BrdU nuclear labeling through subsequent optical sections in the z-axis. 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 5min with a 0.05% solution of 3,38-diaminobenzidine, 0.01% H_2O_2, and 0.04% nickel chloride in TBS (brown-black reaction product).

The cystic lesion size was assessed on serial sagittal Nissl stained sections in 210 µm intervals. Images containing the cystic lesion area were captured at 5x magnification on a Leica DMR microscope with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Michigan, USA). The lesion area was determined by measuring the number of pixels within cystic lesion areas using NIH Image software (version 1.62), which was converted into mm^2. Regenerative responses of CST axons were determined by looking for BDA-labeled and DAB visualized axonal profiles reaching into the cystic lesion center or ventrally around it using a LEICA DMR microscope at 40x magnification in one sagittal section per animal going through the main part of the dorsal CST.

3. RESULTS

3.1 In vitro characterization of adult NPC

The aim of the *in vitro* analysis in the present study was to validate the differentiation pattern in comparison to previous studies [20, 23], and to determine whether retroviral gene transfer may cause a shift in the differentiation pattern of FGF-2 responsive adult NPC. Cells, which were isolated from the adult rat spinal cord at cervical level and plated as monolayers on P-Orn/Lam coated glass coverslips, appear as typical uniform small cells with thin processes (Fig. 1A). Cells maintained in uncoated culture flasks form three-dimensional cell conglomerates (neurospheres) (Fig. 1B). After introducing the reporter gene GFP into NPC using a retroviral vector, which drives GFP expression by a 5' long terminal repeat promoter, transgene expression is detectable *in vitro* over at least 6 passages and a 2 months period (Fig. 1C).

In proliferation conditions using FGF-2, NPC primarily express nestin (19.9% ±

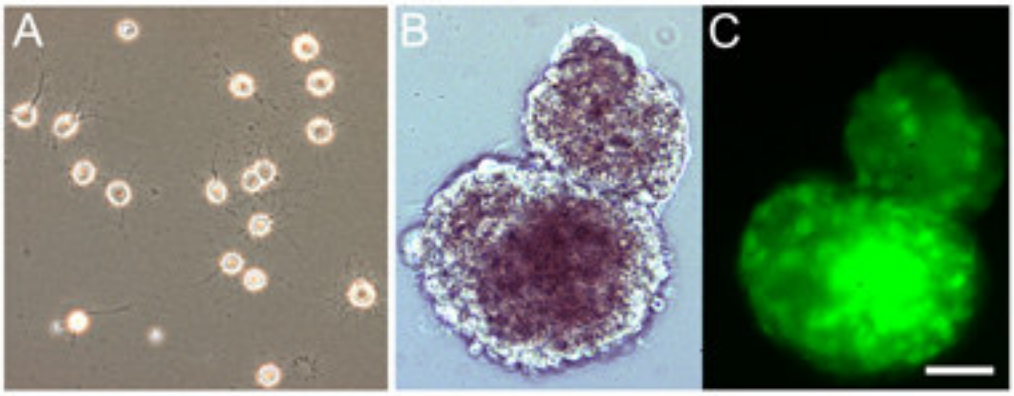

***Figure 1**: Isolation and genetic modification of adult NPC in vitro. NPC isolated from the adult cervical spinal cord were grown (A) as monolayers attached to P-Orn/Lam coated surfaces or (B) as free-floating neurospheres. Phase contrast micrograph. (C) Fluorescence microscopic analysis of the same neurosphere confirms a high transduction efficacy of adult NPC, which have been genetically modified to express GFP using a retroviral vector. Scale bar A 50µm, B, C 75µm.*

6.7) indicating the presence of undifferentiated cells, and the early neuronal marker beta-III-tubulin (18.1% ± 1.7). No cells are found with antigenic properties of astroglia (GFAP expression) or oligodendroglia (GalC expression) (Fig. 2, 3A). After incubation of NPC with 1% FCS instead of FGF-2 (differentiation condition) for one week, nestin immunoreactive cells disappear completely, whereas cells immunoreactive for the oligodendroglial marker GalC increase significantly (15.0% ± 6.5), indicating a shift from undifferentiated towards differentiated cells. The detection of early neuronal antigenicity (beta-III-tubulin) is unchanged in differentiation medium (21.2% ± 3.1). The yield of GFAP expressing cells indicating astroglial differentiation remains low (0.8% ± 0.2), even in differentiation medium (Fig. 2, 3B). Quantitative analysis of cell differentiation after the introduction of the reporter gene GFP through a retroviral vector diluted in serum-containing supernatant reveals no significant changes compared to unmodified NPC in both, proliferation and differentiation conditions. There is a tendency for decreased neuronal antigenicity in GFP transgenic NPC (beta-III-tubulin expression 10.4% ± 3.3), which is not significantly different compared to unmodified NPC (p=0.073) (Fig. 3).

3.2 Survival, proliferation, transgene expression and differentiation of adult NPC in vivo

Adult NPC, either prelabeled with BrdU *in vitro* or genetically modified to express the reporter gene GFP, were transplanted as small neurosphere suspensions right after a cervical wire knife dorsal column transection directly into the injury site. At 3 weeks post injury/transplantation, detailed morphological analysis was performed. NPC readily survive following transplantation into the acutely injured spinal cord as

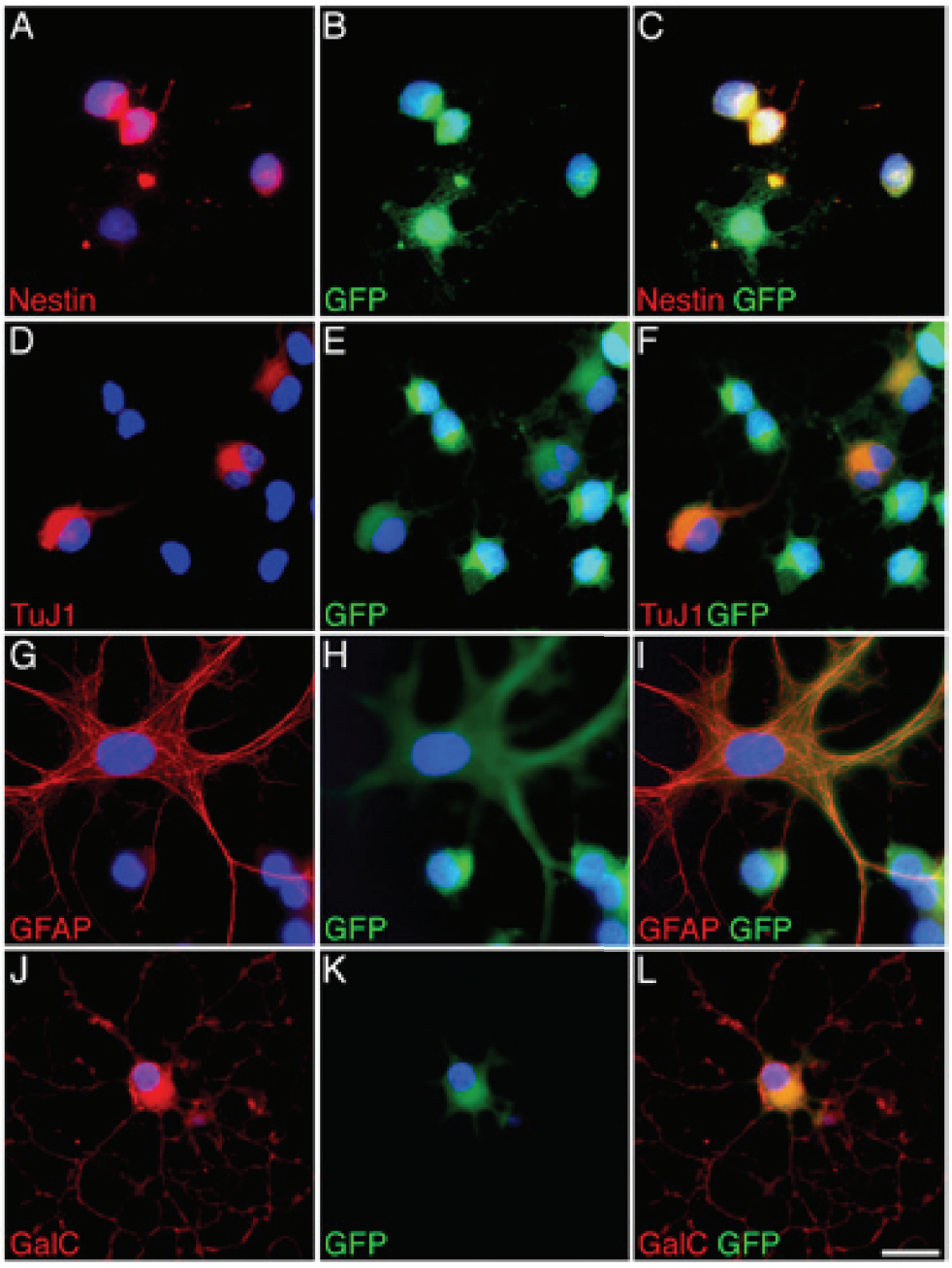

Figure 2: *Differentiation pattern of adult NPC in vitro. The typical immunocytochemical appearance of adult NPC in vitro is exemplified for nestin and beta-III-tubulin in proliferation conditions (FGF-2 added) (A-F), whereas cells expressing GFAP or GalC are shown in differentiation conditions (1% FCS added) (G-L). (A-C) Nestin for neural stem/progenitor cells, (D-F) beta-III-tubulin (clone TUJ1) for neurons, (G-I) GFAP for astroglial cells and (J-L) GalC for oligodendroglia. All differentiation markers are shown in red. Merging of the differentiation markers and GFP (shown in green) confirm the transgene expression in undifferentiated (C) and differentiated cells (F, I, L). Hoechst 33342 as nuclear counterstain (shown in blue). Scale bar A-L 20 µm.*

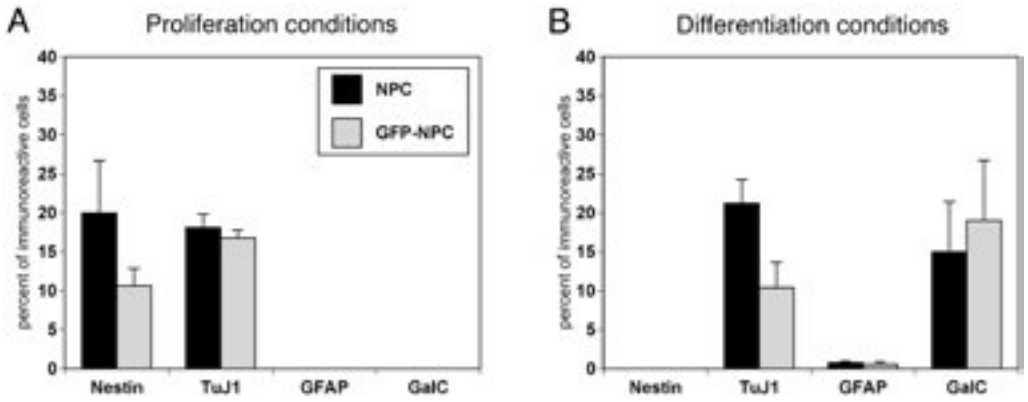

Figure 3: *Quantitative analysis of the differentiation pattern of adult NPC in vitro. (A) Adult NPC maintained in proliferation conditions with FGF-2 or (B) differentiation conditions with 1% FCS. The error bars reflect the standard error of the mean.*

indicated by the widespread detection of BrdU-prelabeled cells. NPC are found predominantly in a dense rim surrounding the cystic lesion and along the central canal (Fig. 4A). NPC detection decreases caudally and rostrally to the lesion site. Cells are found up to 3mm away from the lesion site in the rostral-caudal direction, confirming their migratory capacity. Almost no cells are found ventral to the grafting site in the dorsal column suggesting that migration in the dorso-ventral direction is restricted. BrdU contamination of the NPC containing medium can be excluded, because no BrdU immunoreactivity around the injection site is detectable in control animals receiving injections of supernatant from the last washing step after BrdU incubation into the spinal cord (data not shown). Injection of neural precursor cells, which were killed prior to transplantation by freeze-thaw cycles, did not reveal any unspecific BrdU immunoreactivity in host cells by means of BrdU washed out from dying grafted cells [29]. Another concern is the uncontrolled proliferation of transplanted NPC leading to tumor formation. However, there are no signs of swelling or change of shape of the spinal cord at the transplantation site macroscopically. The low proliferation rate of grafted NPC is confirmed on the molecular level, since very few of the BrdU prelabeled cells are found to co-localize with the proliferation marker Ki-67 3 weeks after the transplantation (Fig. 4A).

A control group of animals, which received GFP expressing and at the same time BrdU-prelabeled NPC (NPC-GFP/BrdU), allowed to assess the stable transgene expression in adult NPC *in vivo* as a prerequisite for the introduction of therapeutic transgenes in subsequent studies. After 3 weeks post transplantation, GFP expression is dramatically downregulated as indicated by the low number of GFP positive cells in contrast to the abundance of BrdU prelabeled transplanted NPC (Fig.4B). The detection of BrdU prelabeled NPC excludes the possibility that a lack

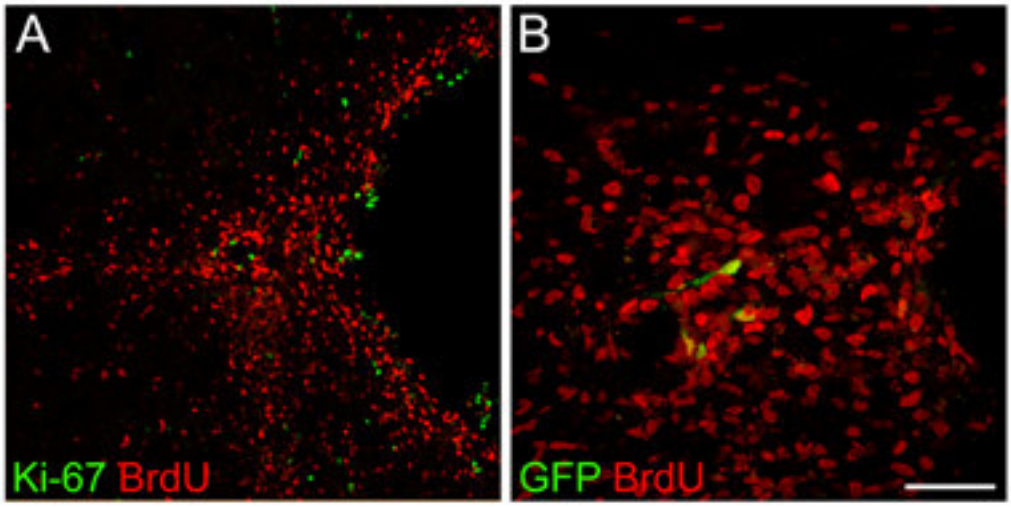

Figure 4: *Survival, proliferation and transgene expression of grafted NPC in vivo. (A) The majority of grafted BrdU prelabeled adult NPC (shown in red) is detected in a rim surrounding the spinal cord lesion site. Grafted cells can be detected in decreasing quantities rostral and caudal up to 2 mm away from the transplantation site. After 3 weeks post transplantation, almost none of the grafted NPC are co-localized with the proliferation marker Ki-67 (shown in green) indicating that the proliferation rate of NPC is rather low after mitogen withdrawal and transplantation (arrowheads highlight the central canal). (B) Almost all grafted BrdU prelabeled NPC (shown in red) lack GFP expression (shown in green) indicating silencing of the transgene in adult NPC 3 weeks after transplantation. Scale bar A 150µm, B 30µm.*

of cell survival is responsible for the poor expression of GFP *in vivo*.

Grafted NPC display antigenic properties of glial precursor cells (NG2), radial glial cells (BLBP), astroglia (GFAP) and oligodendroglia (APC) as confirmed by co-localization with BrdU prelabeled grafted NPC (Fig. 5A-L). None of the grafted NPC are identified to display antigenic properties of neurons (Fig. 5M-O), even though beta-III-tubulin expressing NPC are found frequently *in vitro*.

Most of the differentiation markers used, which are either cytoplasmatic (GFAP, BLBP, APC, beta-III-tubulin) or extracellular (NG2), become upregulated in the host spinal cord displaying a dense immunoreactivity around the injury site. Combined with the fact that BrdU represents a nuclear marker to identify grafted cells, it is impossible to discriminate in all regions unequivocally between co-localization of differentiation markers with grafted NPC versus resident spinal cord cells, which prohibits a thorough quantitative analysis. However as a general observation, the majority of grafted NPC express antigenic properties of radial glia or astroglia, fewer cells show immunoreactivity for oligodendroglial or glial precursor markers.

3.3 Replacement of the cystic lesion defect

One goal of cellular therapy following spinal cord injury is to replace the cystic lesion defect, which represents a typical

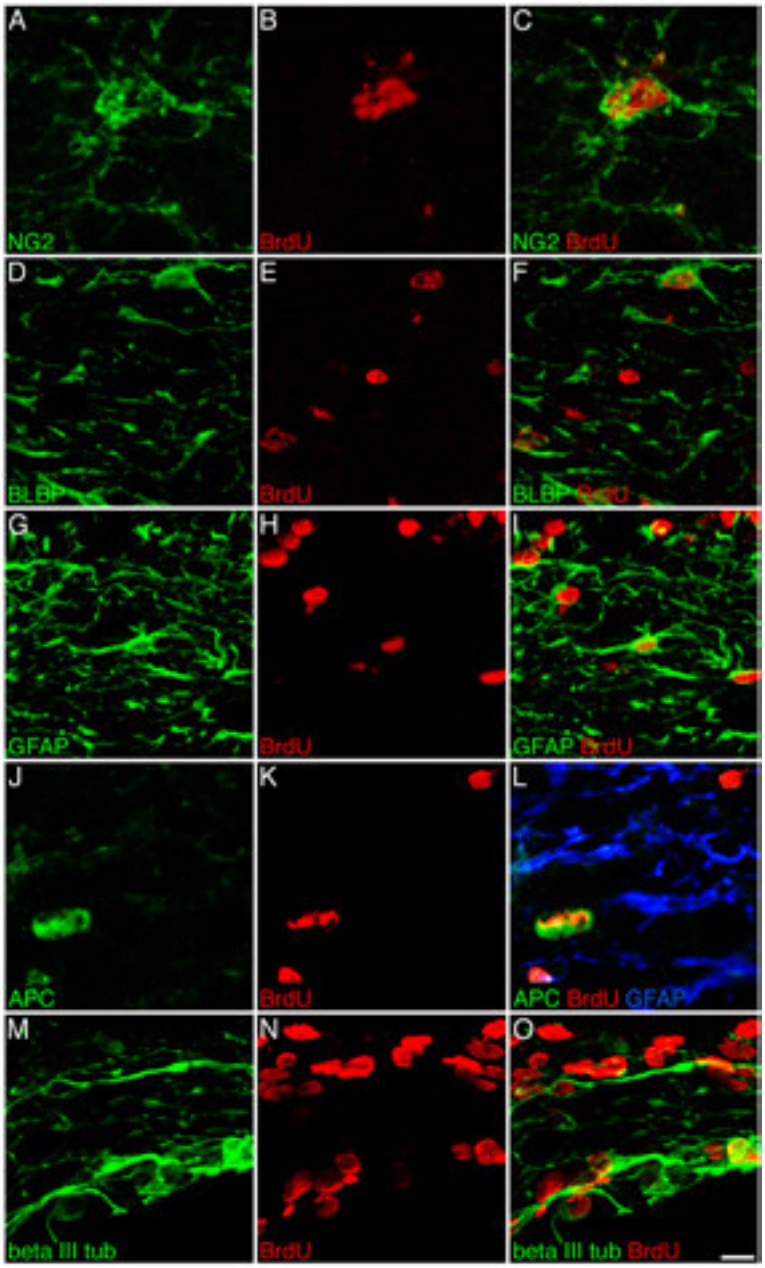

Figure 5: *NPC differentiation in vivo. The differentiation pattern of grafted NPC was assessed by determining the co-localization of individual glial/neuronal markers (shown in green) and BrdU prelabeling (shown in red). Grafted NPC are co-localized with NG2 immunoreactivity representing glial precursor cells (A-C), BLBP immunoreactivity for radial glial cells (D-F), GFAP immunoreactivity for astroglial cells (G-I) and APC immunoreactivity indicating oligodendroglial differentiation (J-L). APC immunoreactive cells do not co-express GFAP (shown in blue) confirming the oligodendroglial differentiation. Adult NPC displaying antigenic properties of neurons (beta-III-tubulin immunoreactivity) are not identifiable (M-O). Confocal fluorescence micrographs, scale bar A-C 5µm, D-O 10µm.*

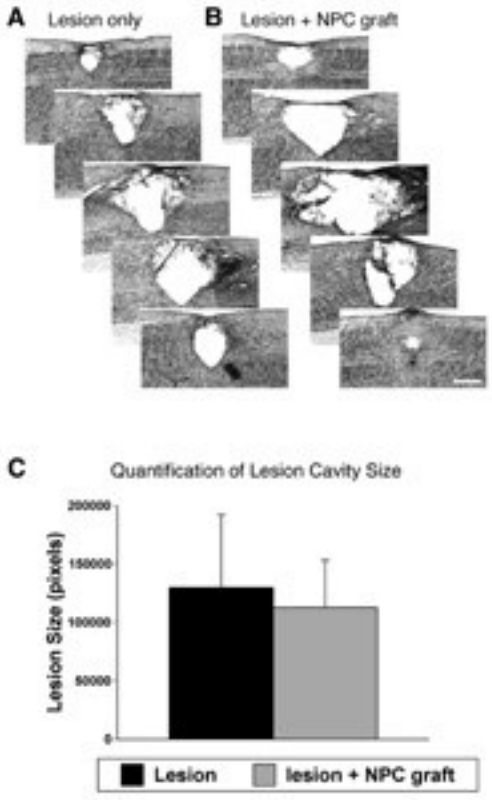

Figure 6: *Lesion size after transplantation of adult NPC. Serial sagittal Nissl stained sections through the spinal cord injury site do not reveal a decrease of the cystic lesion defect after NPC transplantation. (A) Animal with lesions only, (B) animal with a spinal cord lesion followed by NPC transplantation. (C) Quantitative analysis of the cystic lesion size in mm² (error bars represent standard error of the mean). Scale bar A, B 500µm.*

morphological sequel following CNS injury and impedes axonal regeneration. To assess whether grafts of adult NPC would at least partially reduce the lesion area, the cystic lesion compartments were quantified in serial sagittal sections of animals with lesions only versus animals with lesions followed by NPC transplantation. The typical triangular to round shape of a wire knife dorsal column transection does not differ between non-grafted and NPC grafted spinal cord injured animals (Fig. 6A, B). The quantification of the cystic lesion area in consecutive Nissl sections confirmed that, no matter whether animals receive grafts or not, the cystic lesion remains unchanged (Fig. 6C; lesion + NPC graft 0.278mm² ± 0.099; lesion only 0.281mm² ± 0.135).

3.4 Adult NPC grafts and axonal regeneration

In the present study, we performed an incomplete spinal cord injury lesioning specifically the main dorsal CST at cervical level C3. The immunohistochemical analysis of neurofilament expression as an unspecific axonal marker and the visualization of anterogradely BDA-labeled CST axons were employed to determine the spatial association of grafted NPC and injured axons. After 3 weeks of transplantation, processes from GFP expressing NPC are aligned along neurofilament expressing axons adjacent to the cystic lesion area (Fig. 7A). NPC are also identified in between BDA-labeled axonal profiles, with their GFP positive processes appearing to be in contact with CST axons (Fig. 7B). Whether axons regenerate along grafted NPC or whether grafted cells migrate towards these axons and align along them, cannot be determined. Immunohistochemical markers could not further specify the phenotype of these cells, however, morphologically they appear as either astroglia or oligodendroglia. Compared to animals with lesion only, no enhanced regrowth responses of disrupted CST axons in or around the lesion site

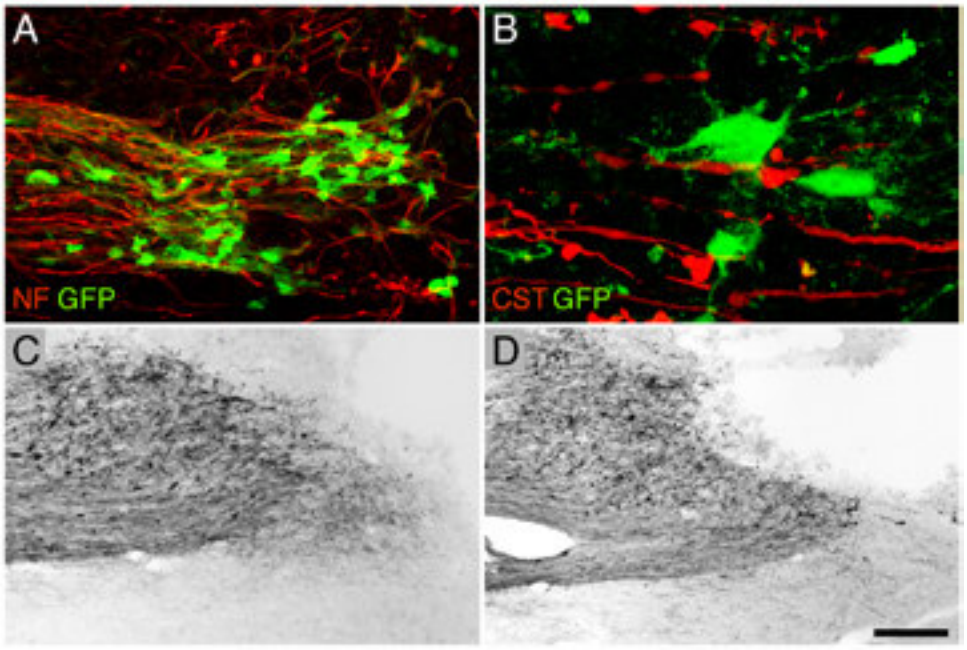

Figure 7: *Grafted NPC and axon regeneration. GFP expressing NPC (shown in green) are located in between neurofilament expressing axons (shown in red) adjacent to the lesion and appear to align along them (A). Similarly, adult NPC are identified to integrate in between BDA labeled CST axons (shown in red) immediately rostral to the lesion site (B). Compared to animals with lesions only (C), animals with lesions and NPC grafts do not reveal an increased regenerative response of interrupted CST axons (D). A,B Confocal immunofluorescence micrographs, C,D brightfield micrographs. Scale bar A 55mm, B 20mm, C and D 200µm*

are observed following NPC transplantation (Fig. 7C, D).

4. DISCUSSION

This study demonstrates that syngenic adult neural progenitors cells survive transplantation into the acutely injured spinal cord, differentiate into oligodendroglia and astroglia and integrate along injured axonal pathways surrounding the lesion defect.

Thus far, studies reported survival of adult NPC only after transplantation into the intact spinal cord [20] or after delayed transplantation into the injured spinal cord [21, 30]. Probably the most inhospitable CNS environment for any cell graft to survive in is an acutely injured area, which is characterized by the initiation of inflammatory cascades, upregulation of mediators of cell death/degeneration and secondary ischemic events [31]. Whether transplantation of NPC as small neurosphere fragments instead of single cell suspensions contributed to the observed graft survival, remains to be determined. Within neurospheres, cell cell contacts remain intact and detrimental effects by dissociation methods to generate single cell suspensions can be avoided [32]. Whether poor cell survival in the fluid-filled lesion cavity or the lack of an extracellular scaffold prevented the cyst replacement cannot be answered in the present study. Find-

ings from studies analyzing the regenerative capacity of embryonic derived neural stem cells, which completely fill out the cystic spinal cord contusion site [18], suggest that adult NPC may indeed produce insufficient extracellular matrix to maintain themselves within the lesion cyst (personal communication, J.W. McDonald).

This study provides important insights into the regenerative potential of adult NPC grafts. Very promising is the observation that adult NPC migrate and align along injured axon pathways caudal and rostral to the lesion site. This finding suggests that they are not sealed off by the surrounding host spinal cord, in contrast to other cell types used for transplantation such as fibroblasts or Schwann cells [9, 13]. Thus, adult NPC may have the capacity to build a continuum between the host and the actual graft. In the present study, there may have been axon sprouting responses around the lesion supported by grafted NPC. However, these effects would have been marginal at best, since thorough qualitative analysis of CST projections did not reveal any differences between lesioned only and NPC grafted animals. Whether adult NPC may provide a cellular scaffold for regenerating axons within the lesion center, and which glial subpopulation may serve as a cellular scaffold to guide growing axons after injury, remains to be determined.

Withdrawal of the mitogen and addition of serum resulted primarily in early neuronal (beta-III-tubulin) and oligodendroglial (GalC) differentiation *in vitro*, in contrast to very few cells expressing astroglial markers (GFAP). The already high proportion of neuronal antigenicity in proliferation conditions remains unchanged in differentiation conditions. It is difficult to validate these data by comparing them with previous studies [20, 23, 24] since differences in cell culture conditions (passage number, density of plated cells in differentiation assays, serum contents) and immunocytochemical analysis make any detailed comparison unreliable. The overall differentiation pattern replicates previous findings, however, we detect a higher proportion of neuronal antigenicity, which might be attributable to the use of Neurobasal medium with B27 supplement [22]. A systematic analysis of different media and supplements influencing the differentiation pattern of subventricular zone derived adult NPC *in vitro* revealed that replacement of B27 supplement by N2 reduces the neuronal antigenicity from 22% to 10%. In line with previous studies describing the differentiation pattern of adult NPC *in vitro* [20, 22], only around one third of NPC display glial or neuronal immunoreactivity. One has to consider that a major proportion of cells has not yet fully differentiated to express marker of mature cells such as GFAP or GalC. This notion is supported by a subsequent experiment, which demonstrates that around 50% of adult NPC *in vitro* represent glial precursor cells expressing A2B5, both in proliferation and differentiation conditions (M. Vroemen, unpublished observation). What becomes clear is that the differentiation pattern *in vitro* has only a very limited predictive value for the differentiation *in vivo*. There, NPC differen-

tiate primarily into astroglial cells, fewer into oligodendroglia, none into neurons 3 weeks after transplantation into the injured spinal cord. As demonstrated by others, molecular cues by the host spinal cord override the differentiation pattern of adult NPC before transplantation [20, 21, 33, 34]. In contrast, embryonic derived neural stem cells, predifferentiated *in vitro* or not, display antigenic properties of glial cells and neurons following transplantation into the spinal cord [17]. It is conceivable that embryonic and adult derived neural stem/progenitor cells show a differential response to instructive cues of the graft environment.

This study reports for the first time oligodendroglial differentiation after transplantation of adult NPC into the injured spinal cord. Apparently, adult spinal cord derived NPC retain the multipotency to differentiate into oligodendroglia and are able to recognize respective instructive signals by the host spinal cord. Oligodendroglial differentiation after transplantation of adult NPC has been shown previously, however, cells were grafted into the intact spinal cord [20]. The only other published study, examining the regenerative capacity of adult heterotopic subventricular zone derived NPC after delayed transplantation into the injured spinal cord, did not observe oligodendroglial differentiation [21]. Differences regarding the region of progenitor cell isolation (spinal cord versus subventricular zone), the propagating conditions *in vitro* (different culture medium or supplements), the timing of transplantation (delayed versus immediately after injury) and the analyzing methods (different antibodies for oligodendroglial differentiation) may all have accounted for the observed differences. The presence of astroglial differentiation and the lack of neuronal differentiation are in accordance with previous studies [20, 21].

The downregulation of transgene expression in transplanted NPC prohibits not only optimal tracking of grafted NPC, but in addition, approaches to introduce therapeutic genes into NPC need to be reconsidered. The phenomenon of downregulation or silencing of transgenes in undifferentiated cells introduced by retroviral vectors has been observed by others [35]. The presence of retroviral silencer elements, cytosine methylation of CpG sites and insufficient promoters are thought to contribute to transgene silencing [35, 36]. More promising approaches to successfully genetically modify adult NPC point to the use of modified promoters or different viral vectors [36-39].

Results from the present study demonstrating, that adult NPC survive transplantation into the acutely injured spinal cord, integrate into the host spinal cord in close spatial association with injured and non-injured CNS axons and differentiate into oligodendroglia and astroglia, are very promising. However, investigations have to be continued before any conclusions regarding the regenerative capacity of adult NPC in spinal cord injury can be drawn. Adult NPC need to fill out the lesion defect to assess their axon growth promoting properties. If there is a regeneration promoting effect, the ideal

cell type, which may provide a scaffold for regenerating axons, has to be identified and specified. The remyelination by grafted adult NPC differentiating into oligodendroglial cells has to be assessed. Strategies need to be developed to enrich favorable glial subpopulations *in vitro*, which maintain their differentiation pattern after transplantation. And finally, considering autologous transplantation of adult neural stem cells as a potential treatment strategy for spinal cord injured individuals, it has to be demonstrated that adult NPC can be harvested from the individual's own CNS, expanded and enriched in culture and re-implanted into the spinal cord lesion site.

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