Cellular therapy after spinal cord injury using neural progenitor cells

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Chapter 4b

Schwann cells fail to replace fibroblasts as supporting cells for adult neural progenitor cell grafts in the acutely injured spinal cord

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ABSTRACT

Adult neural progenitor cells (NPC) co-grafted with fibroblasts replace cystic lesion defects and promote cell-contact mediated axonal regeneration in the acutely injured spinal cord. Fibroblasts are required as a platform to maintain NPC within the lesion, however, they are suspected to create an inhospitable milieu for regenerating CNS axons. Therefore, we thought to replace fibroblasts by primary Schwann cells, which might serve as a superior scaffold to maintain NPC within the lesion and might further enhance axon regrowth and remyelination following spinal cord injury. Adult rats under went a cervical dorsal column transection immediately followed by transplantation of either NPC/Schwann cell or NPC/Schwann cell/fibroblast co-grafts. Animals receiving Schwann cell or fibroblast grafts alone, or Schwann cell/fibroblast co-grafts served as controls. At three weeks after injury/transplantation, histological analysis revealed that only fibroblast containing grafts were able to replace the cystic lesion defect. In both, co-cultures and co-grafts, Schwann cells and NPC were segregated. Almost all NPC migrated out of the graft into the adjacent host spinal cord. As a consequence, only peripheral type myelin, but no CNS type myelin, was detected within NPC/Schwann cell containing co-grafts. Corticospinal axon regeneration into Schwann cell containing co-grafts was massively diminished. Taken together, Schwann cells within NPC grafts contribute to remyelination. However, Schwann cells fail as a supporting platform to maintain NPC within the graft and Schwann cells impair CNS axon regeneration, which makes them an unfavorable candidate to support/augment NPC grafts following spinal cord injury.

1. INTRODUCTION

The development of a cystic lesion cavity represents a typical sequel of spinal cord injury preventing axonal regrowth and functional recovery. In particular, following complete spinal cord injury the introduction of a permissive substrate as replacement for the lesion cavity is required to promote axonal regeneration across the injury site and ultimately reinnervation of target neurons.

Recently, we have demonstrated that adult neural progenitor cells (NPC) not only survive and allow organotypic cell replacement after transplantation into the acutely injured spinal cord, but also replace cystic lesion defects and promote cell-contact mediated regeneration of the corticospinal tract (CST) after co-grafting with primary fibroblasts [1, 2]. Fibroblasts were required to provide a supporting scaffold to maintain adult NPC within the lesion cavity. If one considers NPC transplantation for clinical use, fibroblasts need to be replaced by a more suitable cellular or non-cellular supporting scaffold, since there are major concerns that fibroblasts and extracellular matrix molecules released by fibroblasts represent potent inhibitors of axonal regrowth in the mammalian cen-

tral nervous system (CNS) [3, 4].

Schwann cells might represent an ideal cell population to be combined with NPC instead of fibroblasts for transplantation. Schwann cells are the key factor to support spontaneous axonal regeneration following peripheral nerve injury [5]. Even after transplantation into the injured spinal cord, Schwann cells have been shown to promote axon regrowth and remyelination [6-8]. Furthermore, Schwann cells intrinsically produce favorable extracellular matrix molecules such as laminin [9], which might be suitable as a supporting scaffold for co-grafted adult NPC. Schwann cells intrinsically secrete growth promoting neurotrophic factors and, as opposed to NPC [10], Schwann cells can be easily genetically modified to overexpress therapeutic transgenes [11-13].

In the present study we thought to elucidate whether Schwann cells instead of fibroblasts might provide a more favorable cell-based supporting scaffold for co-grafted adult NPC, which would allow superior structural repair as prerequisite for substantial functional recovery. Schwann cells were co-grafted with adult NPC into acute cervical dorsal column transections in adult rats. Results demonstrate that Schwann cells alone are not sufficient to maintain co-grafted adult NPC in the lesion cavity. Only the addition of fibroblasts to Schwann cell containing grafts allows cyst replacement. However, Schwann cell containing grafts dramatically impair CNS axon regeneration.

2. MATERIALS AND METHODS

2.1 Animals

Adult female Fischer 344 rats (160-180g) were used. All experiments were carried out in accordance with the institutional guidelines for animal care. All efforts were made to minimize the number of animals used. Animals had *ad libitum* access to food and water throughout the study.

All surgical procedures were performed under anesthesia with a combination of ketamine (62.5mg/kg body weight; WDT, Garbsen, Germany), xylazine (3.175mg/kg body weight; WDT) and acepromazine (0.625mg/kg body weight, Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution.

2.2 Cell preparation

For the isolation of NPC, fibroblasts and Schwann cells, adult female Fischer 344 rats were deeply anesthetized as described above and killed by decapitation. NPC were isolated from the cervical spinal cord as described before [1]. Briefly, the cervical spinal cord (spinal cord level C3 through T1) was dissected out, minced and washed in sterile Dulbecco's phosphate buffered saline/D-glucose (PBS, 4.5g/l; PAA Laboratories, Linz, Austria). Subsequently, the tissue was 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 MgSO4, dissolved in Hank's balanced salt solution (HBSS; PAA Laboratories) for 30 min at 37°C. The

resulting cell suspension was washed and plated on culture dishes containing serum-free growth medium, consisting of Neurobasal medium with B27 supplement (Gibco) and 20 ng/ml recombinant human FGF-2 (R&D System, Wiesbaden, Germany). Cells were expanded either as neurospheres in uncoated culture flasks or as adherent monolayer in culture flasks coated with poly-l-ornithine (20µg/cm^2; Sigma) and laminin (0.4µg/cm^2; Sigma) as described before [1].

Primary fibroblasts were isolated from adult Fischer 344 rat skin biopsies and cultured in DMEM medium (Pan Biotech, Aidenbach, Germany) supplemented with 10% of FCS as described before [14].

Schwann cells were isolated from adult sciatic nerve biopsies employing magnet-activated cell separation (MACS) of p75 low affinity NGF receptor expressing Schwann cells according to established protocols [15]. The purity of Schwann cells, which was assessed by comparing the number of Hoechst stained nuclei with p75 low affinity NGF receptor expressing cells, was >99%. Purified Schwann cell cultures were expanded in DMEM medium supplemented with 10% FCS, 2 mM forskolin (Sigma) and 0.2% bovine pituitary extract (Clonetics, Oldendorf, Germany).

For the identification of co-cultured NPC *in vitro* and co-grafted Schwann cells *in vivo*, the respective cell populations were genetically modified to express the reporter gene green-fluorescent protein (GFP) using a MLV-based retroviral vector pCLE-GFP containing the internal EF-1alpha promoter [1, 10]. NPC or Schwann cells (passage 3-5) were plated in sub-confluent densities and were exposed on two consecutive days with retrovirus containing growth medium supplemented with 1 µg/ml Polybrene (Sigma). G418 (500µg/ml; Gibco) was added to the growth medium for the selection of cells, which integrated the retroviral vector. Successful incorporation of the transgene was confirmed by detection of GFP using an inverted fluorescence microscope (Olympus IX 70).

2.3 Co-cultures of adult NPC and Schwann cells

For the analysis of NPC differentiation *in vitro*, GFP expressing NPC maintained as neurospheres were dissociated using Accutase (Innovative Cell Tech, San Diego, USA) and incubated in NB medium with B27 supplemented with 5% FCS for 7 days after seeding onto poly-l-ornithine/laminin (Sigma) coated cover slips (Pan Biotech). For the analysis of NPC differentiation co-cultured with Schwann cells, GFP expressing NPC were seeded onto a confluent layer of Schwann cells. Co-cultures were incubated for 7 days in NB medium with B27 supplemented with 5% FCS. Cells were fixed with 4% paraformaldehyde in PBS and immunolabeled using the following antibodies: 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-A2B5 for glial precursor cells (Chemicon; at 1/100), mouse-anti-RIP for oligoden-

droglia (Chemicon; at 1/500), mouse-anti-beta-III-tubulin for neurons (Promega; at 1/500) and rabbit-anti-GFP for GFP expressing cells (Molecular Probes, Leiden, The Netherlands; at 1/1000). Cells were washed three times with tris-buffered saline (TBS) after fixation, 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 Alexa-568 linked secondary goat-anti-mouse (Molecular Probes; at 1/1000) and Alexa-488 linked goat-anti-rabbit secondary antibodies (Molecular Probes; 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). 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). The number of specifically immunolabeled cells and the total cell number (number of Hoechst counterstained nuclei) were determined in 5 random fields for each differentiation marker to determine the percentage of cells expressing the respective differentiation marker. Three independently performed immunocytochemical stains were analyzed under each condition.

2.4 Preparation of cells for transplantation

For the identification after transplantation, NPC were prelabeled with the thymidine analogue bromodeoxyuridine (BrdU; Sigma) 48h before transplantation as described [1]. Expression of the reporter gene GFP allowed to identify Schwann cell grafts *in vivo*. Immediately before transplantation, respective cells were harvested and washed three times in PBS. A sample of the resulting single cell suspensions was stained with Trypan Blue (Sigma) and counted using a Neubauer hemocytometer. NPC, Schwann cell and fibroblast suspensions were mixed and diluted in PBS to yield the appropriate cell densities in the different grafting paradigms (Table 1). Due to the larger cell size of fibroblasts in comparison to Schwann cells and NPC, the cell concentration in different grafting conditions was adapted to yield equally dense suspensions.

2.5 Surgical procedures

Spinal cord lesions, cell transplantation and anterograde labeling of the CST were performed as previously described [1, 2]. 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 2.5 µl cell suspension (for different grafting conditions and experimental groups see Table 1) was injected directly into the lesion site through a pulled glass micropipette (100 µm internal diameter)

Table 1: *Cell concentration in different grafting conditions:*

Experimental group		Fibroblasts	Schwann cells	Neural progenitor cells
FF	(n=10)	1.5 x 10^5/µl	--	--
SC	(n=8)	--	3 x 10^5/µl	--
NPC/SC	(n=7)	--	1.25 x 10^5/µl	2.5 x 10^5/µl
SC/FF	(n=6)	1.5 x 10^4/µl	3 x 10^5/µl	--
NPC/SC/FF	(n=6)	6.3 x 10^3/µl	1.25 x 10^5/µl	2.5 x 10^5/µl

using a Picospritzer II (General Valve, Fairfield, USA). Animals receiving a dorsal column transection without cell transplantation served as control (**Lesion**; n=6). For anterograde tracing of the CST, 300 nl of a 10% solution of biotinylated dextran-amine (BDA; 10.000 MW, Molecular Probes) 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 (General Valve) 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 and *in vivo* differentiation pattern. The following primary antibodies were used: mouse-anti-GFAP for astrocytes (DAKO; at 1/200), mouse-anti-APC for oligodendrocytes (Oncogene, Darmstadt, Germany; at 1/1000), rabbit-anti-NG2 for glial precursor cells (Chemicon; at 1/200), goat-anti-doublecortin for neuronal precursor cells (Santa Cruz Laboratories, Santa Cruz, USA; at 1/500), rabbit-anti-200 kDa neurofilament as general axonal marker (Boehringer Mannheim, Germany; at 1/250), mouse-anti-P0 for peripheral myelin (generous gift from J. Archelos, University of Duesseldorf, Germany; at 1/500), mouse-anti-myelin oligodendrocyte-specific protein (MOSP) for CNS myelin (Chemicon; at 1/5000), rat-anti-BrdU for BrdU-prelabeled cells (Harlan SeraLab, Loughborough, UK; at 1/500) and rabbit-anti-GFP for GFP expressing cells (Molecular Probes; at 1/1000). Immunolabeling with primary antibodies was visualized using Alexa-488, Alexa-568 or Alexa-660 linked donkey secondary antibodies (Molecular Probes; 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 fluorophore (Alexa-488/-568/-660) conjugated secondary donkey antibodies (Molecular Probes; at 1/1000) for 2.5 h. BDA labeled CST axons were visualized by incubation with Cy5-conjugated streptavidin (Jackson; at 1/1000). After a final rinsing step in TBS, sections were mounted onto glass slides and coverslipped with ProLong Antifade Kit (Molecular Probes). Confocal immunofluorescence microscopy was performed using a Leica TCS-NT system.

2.7 Quantification of lesion size and axon regrowth

The investigator quantifying cavity size and axonal regeneration was blinded for the group identity of each analyzed section. The lesion cavity and graft sizes were quantified by surrounding respective structures with a digital pen in a 1 out of 7 series of Nissl stained sagittal sections of each animal using the NIH Image J 10.2 software. For the quantification of axon (neurofilament immunoreactive axonal profiles) and CST axon growth (BDA labeled axonal profiles) within the graft, one sagittal section per animal through the main portion of the respective graft was chosen. 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. 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. The number of pixels representing the labeled axons was assessed using NIH Image J software.

2.8 Statistical analysis

All data are expressed as mean ± SEM. Comparisons of NPC cultures with SC/NPC co-cultures were made by using a Student's T-test. Comparisons of cyst size and axonal regeneration were made using the Kruskal-Walis nonparametric analysis of variance, followed by Dunn's multiple comparison *post hoc* testing. For all statistical tests, $p<0.05$ was considered significant.

3. RESULTS

3.1 In vitro analysis of NPC/Schwann cell interactions.

The aim of the present study was to investigate a transplantation paradigm, where adult NPC are combined with Schwann cells representing a more growth conducive cell population as opposed to fibroblasts, which have been employed in a previous study [2]. Before transplantation, we first investigated the interaction of Schwann cells and NPC (changes in differentiation, cell adhesion) *in vitro*. NPC were co-cultured with Schwann cells un-

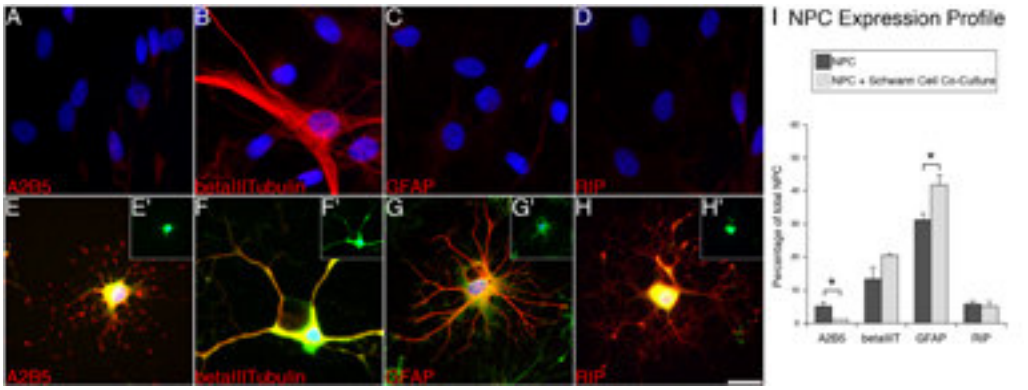

Figure 1: *Primary Schwann cells induce glial differentiation in NPC in vitro. The differentiation markers for NPC, (A, E) A2B5 for glial precursor cells, (B, F) beta-III-tubulin for neurons, (C, G) GFAP for astroglia and (D, H) RIP for oligodendroglia (all shown in red), were investigated for their cross-reactivity with primary Schwann cells (A-D). Of these markers, Schwann cells strongly expressed beta-III tubulin (B), whereas GFAP immunoreactivity was rather weak (C). Reporter gene (GFP) expressing NPC (green) are immunoreactive for A2B5, beta-III tubulin, GFAP and RIP (E-H). (I) The quantification of respective immunocytochemical stains revealed that co-culturing of NPC and Schwann cells significantly increased the proportion of GFAP expressing astroglia, while A2B5 expressing glial precursor cells were significantly reduced ($p<0.05$). A-H Hoechst 33342 as nuclear counterstain (blue). Epifluorescence micrographs. Scale bar A-H 20µm.*

der NPC differentiation conditions for 7 days as previously described [2, 10]. Unlike fibroblasts, Schwann cells do not attach to uncoated cell culture surfaces. Therefore, Schwann cells were plated on poly-l-ornithine/laminin coated glass coverslips and grown until confluent in NPC differentiation medium (NB medium supplemented with B27 and 5% FCS). Subsequently, undifferentiated NPC transduced to express the reporter gene GFP (GFP-NPC) were plated onto a Schwann cell monolayer and incubated for 7 days in NPC differentiation medium. Since Schwann cells have been described to express glial and neuronal markers relevant for NPC differentiation [16, 17], we first checked for cross-immunoreactivity of Schwann cells for the NPC differentiation markers A2B5, beta-III-tubulin, GFAP and RIP (Fig. 1A-D). Schwann cells displayed only strong immunoreactivity for beta-III-tubulin, which is commonly used to indicate early neuronal differentiation in NPC. However, beta-III-tubulin positive Schwann cells were clearly distinguishable by their prominent nucleus and compact morphology as opposed to rather small nuclei and fine processes of beta-III-tubulin immunoreactive NPC. Co-cultured NPC were immunoreactive for markers identifying glial precursor cells (A2B5 expression), astroglia (GFAP), oligodendroglia (RIP) and neurons (beta-III-tubulin) (Fig. 1E-H) in accordance with previous studies [1, 2]. The expression of these markers in NPC was quantified to detect a potential influence of co-cultured Schwann cells on the differentiation pattern of NPC (Fig.1I). The presence of Schwann cells significantly

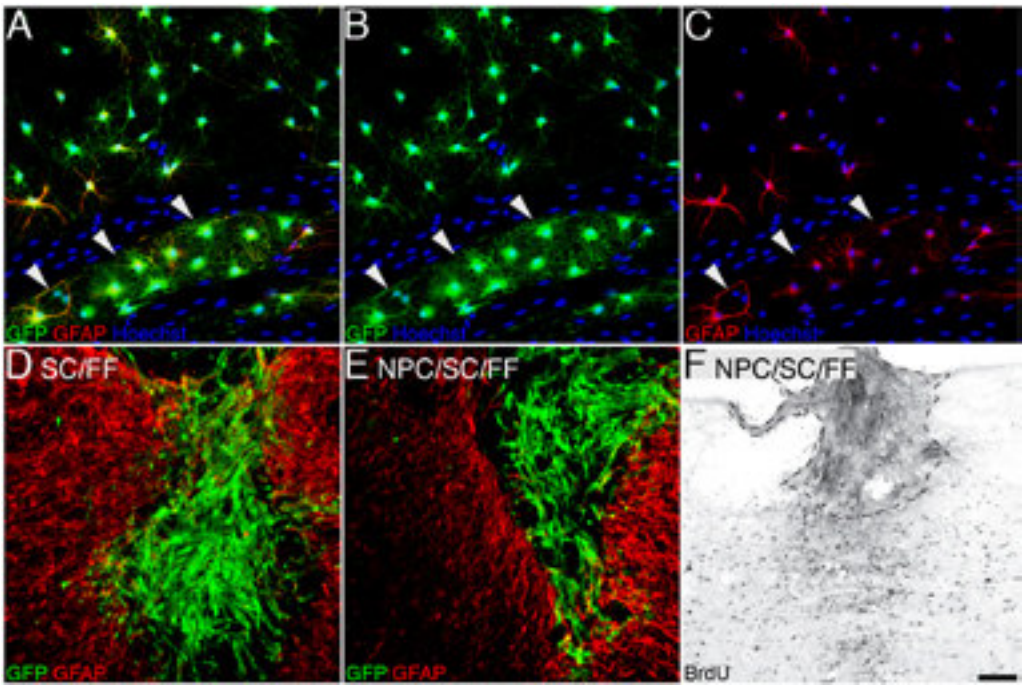

***Figure 2**: NPC and Schwann cells become segregated in vitro and in vivo. (A) After 7 days of co-culturing Schwann cells and GFP expressing NPC (green) in differentiation medium, NPC and Schwann cells (identified by GFP-negative Hoechst 33342 counterstained nuclei) appear completely segregated on the poly-l-ornithine/ laminin coated cell culture surface. GFAP positive processes (red) co-localized with GFP expressing NPC seal off the NPC from the Schwann cell area (arrowheads). Overlay GFP (green), GFAP (red) and Hoechst 33342 (blue). (B) GFP (green) and Hoechst 33342 (blue). (C) GFAP (red) and Hoechst 33342 (blue). (D) SC/FF grafts fill out the lesion defects with GFP-expressing Schwann cells (green) densely distributed throughout the graft. Doublelabeling with GFAP (red) indicates that the SC/FF graft is delineated from the adjacent host parenchyma by a astroglial border. (E) Identical situation with NPC/SC/FF grafts. GFP-expressing Schwann cells (green), GFAP (red). (F) Immunohistochemical detection of BrdU prelabeled NPC reveals that the vast majority of NPC has migrated from the NPC/SC/FF graft (dashed line) into the surrounding host spinal cord. A-C epifluorescence micrographs, D, E confocal fluorescence micrographs, F brightfield micrographs. Orientation in D-F: rostral is to the left, dorsal to the top. Scale bar A-C 50 µm, D, E 73 µm and F 87 µm.*

reduced the proportion of A2B5 positive glial precursor cells (5% ± 1.3 versus 1.1% ± 0.1), whereas the proportion of GFAP positive astroglia was increased from 31.3% (± 1.8) in the NPC differentiation condition compared to 41.8% (± 2.9) in the Schwann cell-NPC co-culture differentiation condition. Beta-III tubulin and RIP immunoreactivity was not altered by co-cultured Schwann cells.

After 7 days of co-culture, NPC, which were initially plated onto Schwann cell monolayers, were completely separated from Schwann cells (Fig. 2A-C). GFAP-positive astrocytes with a stellate morphology reminiscent of reactive astroglia appear to seal off the ‘NPC compartment’ from the ‘Schwann cell compartment’, whereas Schwann cells represented by GFP negative Hoechst stained nuclei are

arranged in compact streams in between patches of GFP positive NPC.
Taken together, Schwann cells induced a shift of adult NPC from immature to mature astroglial differentiation *in vitro*. The segregation of co-cultured NPC and Schwann cells suggests that a homogenous distribution of these cell populations after transplantation *in vivo* is unlikely.

3.2 Cyst replacement, cell survival and differentiation of grafted NPC.

The size of the cystic lesion defect in the various grafting conditions (Table 1) was quantified, to determine the capacity of Schwann cells to maintain co-grafted NPC within the cystic lesion environment. Animals with dorsal column transsections at cervical level receiving either 1) fibroblasts only **(FF)**, 2) highly purified Schwann cells only (**SC**), 3) NPC and Schwann cells (**NPC/SC**), 4) fibroblasts and Schwann cells (**SC/FF**) or 5) NPC, Schwann cells and fibroblasts (**NPC/SC/FF**) were compared with lesioned only animals (**Lesion**). Without grafting, the described spinal cord lesion produces a cystic lesion cavity within the dorsal column measuring 3.68 ± 0.62 mm ^{2} (Fig. 3A,G). Schwann cell grafts alone (SC) or combined NPC/Schwann cell transplants (NPC/SC) allowed only a cyst reduction between 53.5 and 46.2% of its original size (Fig. 3C,D,G). Increasing the number of transplanted Schwann cells in these conditions did not further diminish the lesion defect (data not shown). Only the addition of primary fibroblasts (5% of the total number of grafted Schwann cells) dramatically improved the cyst replacement reducing the lesion size by 89.4% (SC/FF), 81.5% (NPC/SC/FF) and 88% (FF) (Fig. 3B,E,F,G).
Both regeneration promoting cell populations, Schwann cells and NPC, survived after transplantation into the acutely injured spinal cord (Fig. 2D-F). Schwann cells expressing the reporter gene GFP were restricted to the former cystic lesion area without migration into the surrounding host parenchyma in respective co-grafting conditions, whereas BrdU-prelabeled NPC were identified primarily in the spinal cord adjacent to the graft (NPC/SC/FF) with few NPC within the actual graft. Thus, cystic lesion replacement is mainly accomplished by grafted Schwann cells supported by fibroblasts.
The pattern of Schwann cell/NPC distribution *in vivo* recapitulates the *in vitro* finding, where Schwann cells and NPC become segregated spontaneously within a short period of time (Fig. 2A-C). The lining of GFAP positive astroglial processes along Schwann cells streams *in vitro* is paralleled by a strongly GFAP immunoreactive astroglial rim surrounding Schwann cell containing co-grafts *in vivo* (Fig. 2D,E).
In parallel with previous observations, the co-grafted NPC mainly differentiated into GFAP positive astroglia (Fig. 4A, B), fewer GFAP negative grafted cells were also found to express APC indicating oligodendoglial differentiation (Fig. 4C). Rarely, NPC were immunoreactive for NG2, which is expressed on

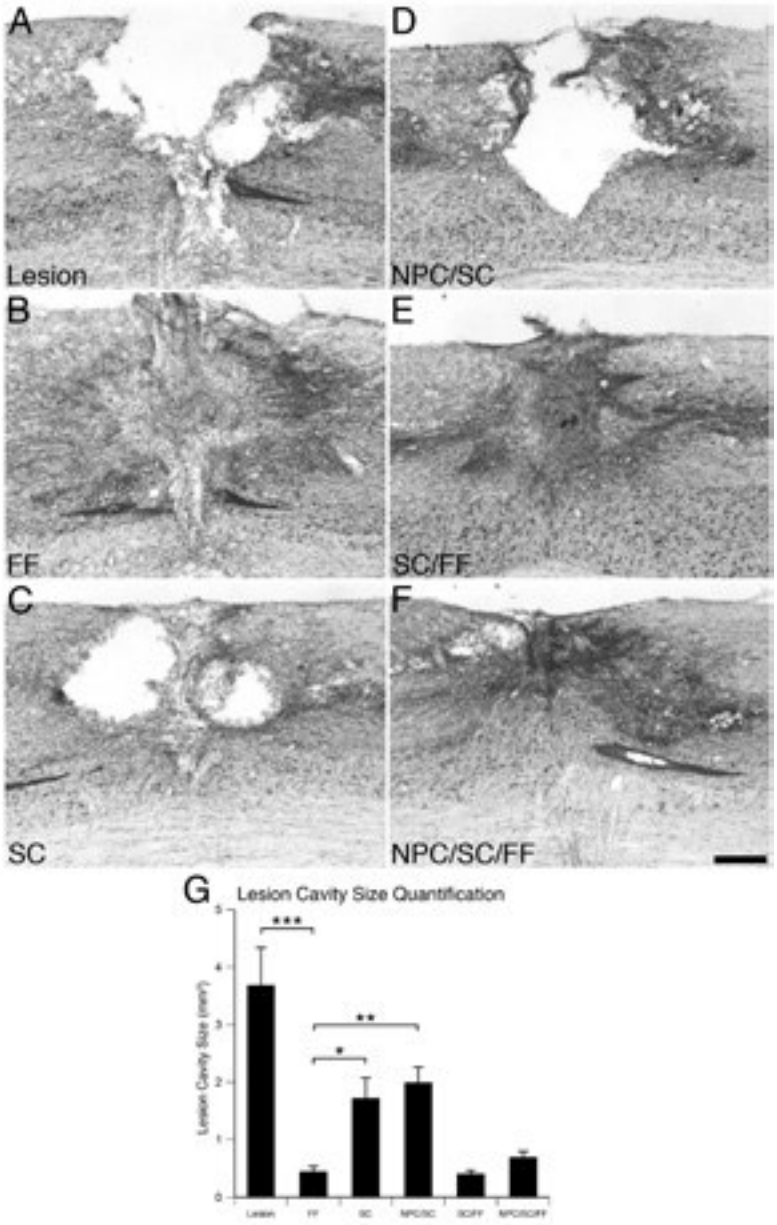

Figure 3: *Sufficient cyst replacement requires fibroblast containing grafts. (A) A cervical wire knife dorsal column transection typically results in a large cystic lesion defect without therapeutic intervention. (B) Pure fibroblasts grafts (FF) replace the lesion cyst, whereas (C) highly purified Schwann cells (SC) or (D) combined grafts of NPC and Schwann cells (NPC/SC) leave the majority of the lesion defect unchanged. As soon as 5% fibroblasts are added to (E) Schwann cell grafts (SC/FF) or (F) combined NPC and Schwann cell grafts (NPC/SC/FF) the lesion defect is almost completely replaced. (G) Quantification of the cystic lesion area in each grafting paradigm compared to animals with lesions only (Lesion). (** indicates p<0.01, *** indicates p<0.001). A-F Brightfield micrographs of sagittal Nissl-stained sections. Orientation A-F: rostral to the left, dorsal to the top. Scale bar A-F 400µm.*

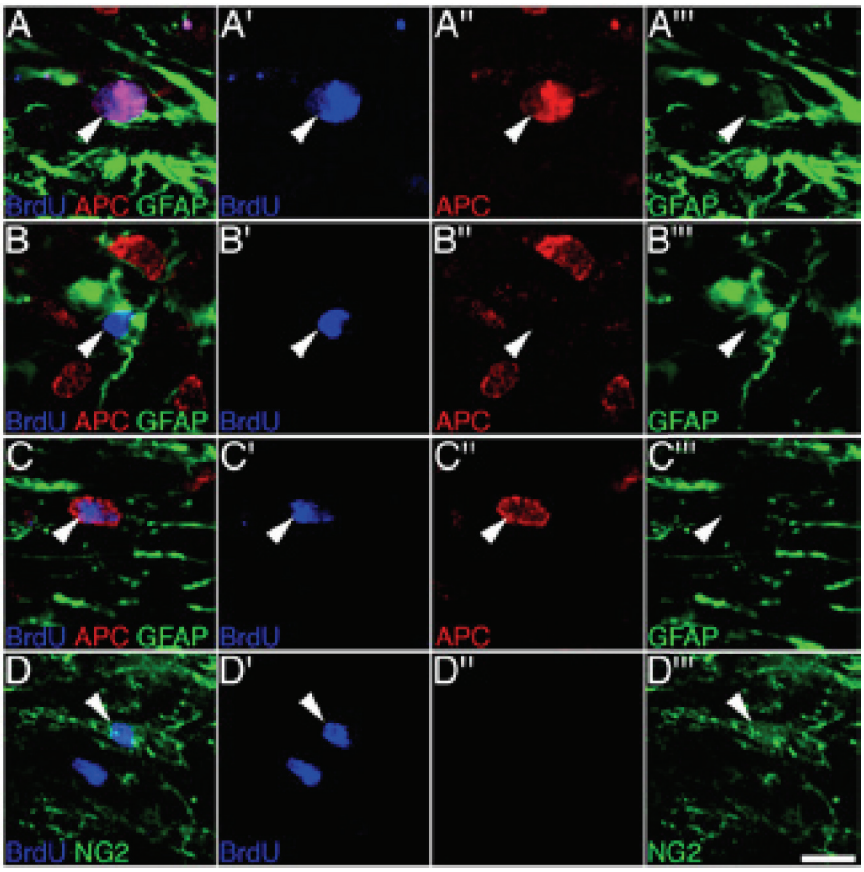

Figure 4: *NPC combined with Schwann cells differentiate into astroglia and oligodendroglia in vivo. The differentiation pattern of grafted NPC was assessed by identifying respective differentiation markers (red and green) co-localized (arrowheads) with BrdU prelabeled NPC (blue) within the adjacent spinal cord parenchyma. Grafted NPC co-localized with (A) APC and GFAP, or (B) GFAP alone indicate astroglial differentiation, whereas (C) co-localization with APC, but not with GFAP, depicts oligodendroglial differentiated NPC. (D) Few grafted NPC could be matched with NG2, a marker for glial restricted progenitor cells. Confocal fluorescence micrographs. Orientation in all micrographs rostral to the left, dorsal to the top. Scale bar A-D 10 µm.*

immature glial cells, 3 weeks after transplantation (Fig. 4D). As expected, none of the grafted NPC displayed the early neuronal differentiation marker doublecortin *in vivo* (data not shown).

3.3 Axonal response

As shown above, only fibroblast containing transplants sufficiently replaced the cystic lesion defect as a prerequisite to build a bridge for axons to regenerate on. Three weeks after lesioning and grafting the axonal response into the respective grafts was assessed quantifying 1) neurofilament immunoreactive axons and 2) specifically anterogradely BDA labeled corticospinal axons. Co-grafts containing NPC and Schwann cells (NPC/SC/FF) elicited the highest overall axonal regrowth (6366 ± 841 pixels/mm ^{2}) com-

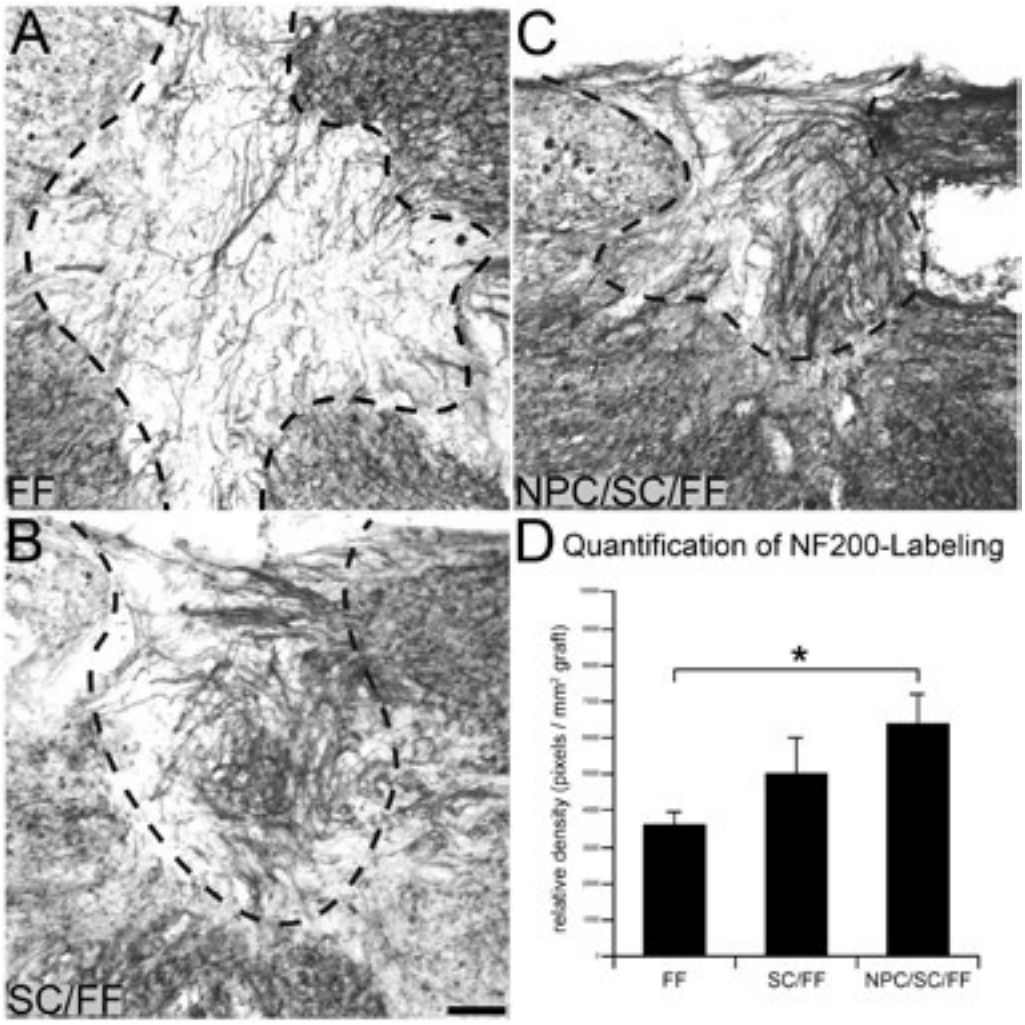

Figure 5: *Axonal growth is enhanced into Schwann cell containing grafts. Immunohistochemical analysis of 200kD neurofilament expression reveals that the density of neurofilament expressing axons within respective grafts increases from (A) FF over (B) SC/FF and (C) NPC/SC/FF grafts, which is confirmed by (D) quantification of neurofilament density (* indicates $p<0.05$). A-C dashed lines outline the graft/host border. Brightfield micrographs. Orientation in A-C: rostral to the left, dorsal to the top. Scale bar A 125 µm, B, C 100 µm.*

pared to co-grafts with Schwann cells (SC/FF; 4998 ± 1008 pixels/mm ^{2}) and pure fibroblasts grafts (FF; 3632 ± 347 pixels/mm ^{2}) as assessed by the density of neurofilament positive axonal profiles (Fig. 5). Analysis of neurofilament expression does not allow to distinguish between axonal regrowth from CNS derived axons versus ingrowth from peripheral nerve derived axons through the dorsal root entry zone. Therefore, corticospinal axons were specifically anterogradely labeled using BDA and quantified (Fig. 6). The degree of corticospinal axon regrowth was significantly reduced in Schwann cell containing co-grafts

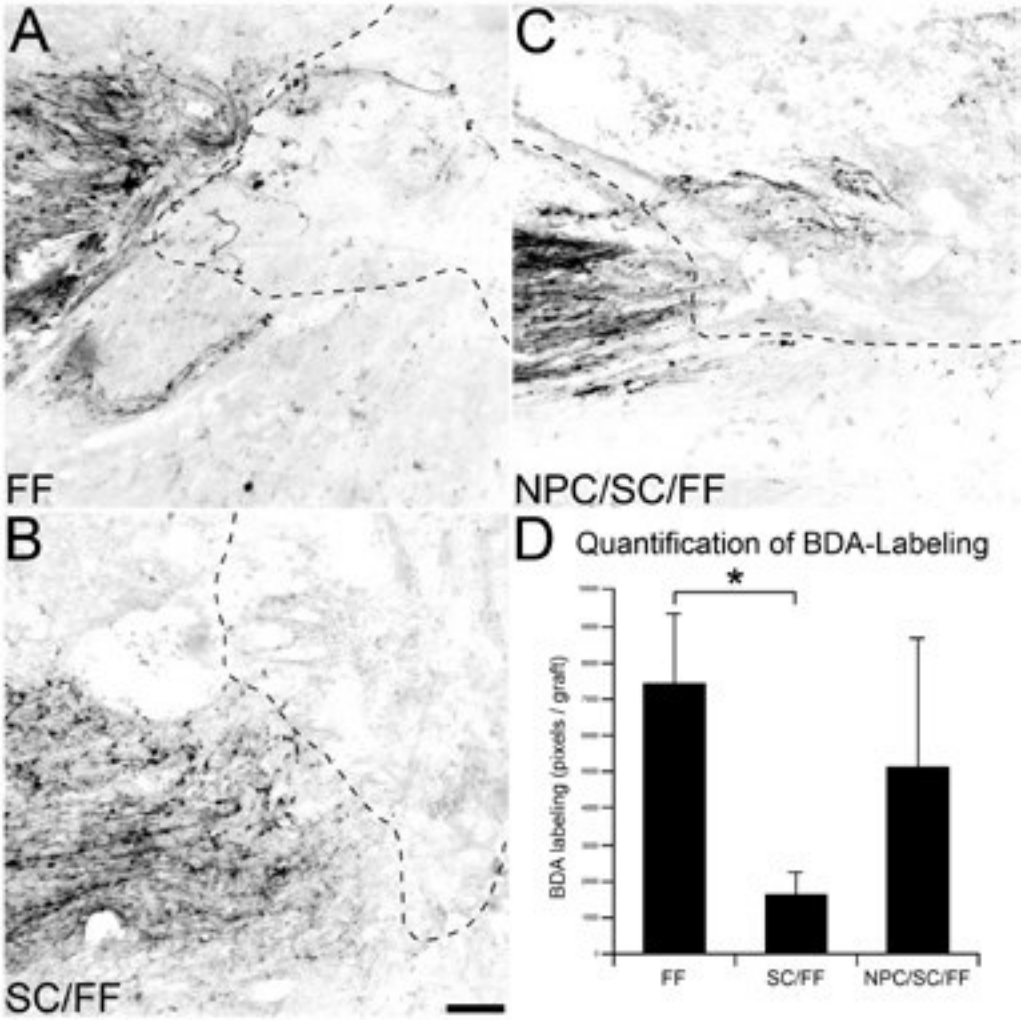

Figure 6: *Schwann cell containing grafts impair corticospinal axon regeneration. Brightfield microscopical analysis of anterogradely BDA-labeled dorsal corticospinal axons shows the regenerative response of CNS axons into (A) FF (B) SC/FF and (C) NPC/SC/FF grafts. Dashed lines outline the graft/host interface. (D) As determined by quantification of BDA labeled corticospinal axon profiles, CST regeneration is massively reduced in animals with SC/FF grafts. (*indicates p<0.01). A-C brightfield micrographs. Orientation in A-C: rostral to the left, dorsal to the top. Scale bar A-C 100 µm.*

(SC/FF; 1601 ± 628 pixels) compared to pure fibroblast grafts (FF; 6879 ± 1797) (Fig. 7A,B,D). NPC/SC/FF co-grafts did not significantly alter the degree of CST axon regeneration (Fig. 7C,D; 5105 ± 3553). Thus, as soon as Schwann cells are added to the grafting paradigm, CST regeneration is dramatically impaired. By combining Schwann cells with NPC, this impaired regeneration can be reversed, but only up to a degree of axonal regeneration observed with pure fibroblast grafts.

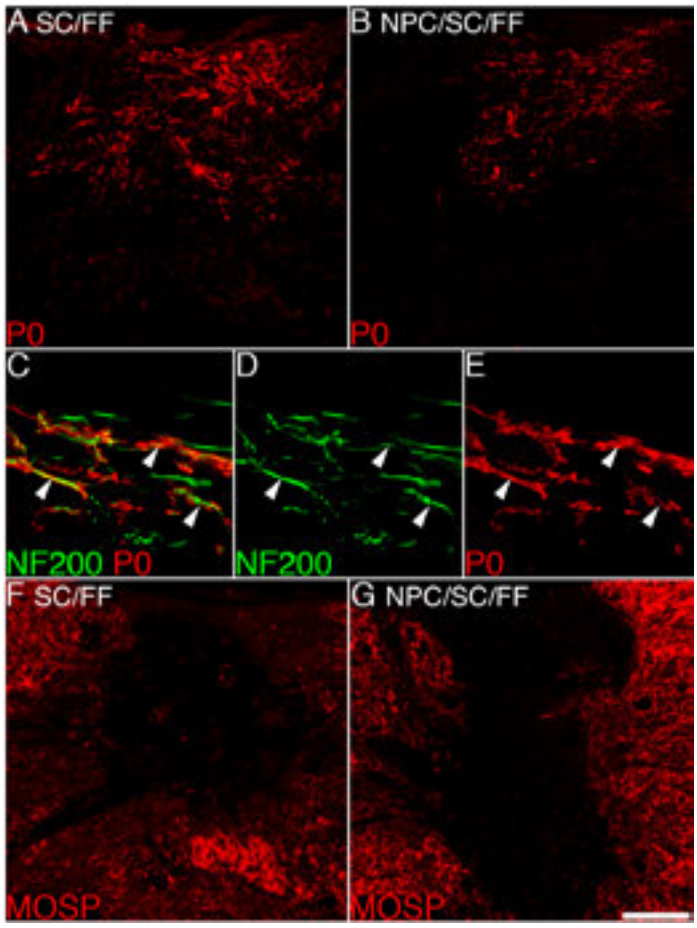

***Figure 7** : Only peripheral type myelin can be identified within co-grafts. PNS myelin indicated by P0 immunoreactivity (red) is abundant throughout Schwann cell containing grafts such as (A) SC/FF and (B) NPC/SC/FF grafts. (C) P0 (red) and neurofilament immunoreactive (green) structures within Schwann cell containing grafts display a close spatial correlation (arrowheads), which indicates remyelination of regenerating axons by grafted Schwann cells. Overlay. (D) Neurofilament. (E) P0. (F) MOSP immunoreactivity representing CNS myelin was virtually absent both in co-grafts without (SC/FF) and co-grafts with (NPC/SC/FF) NPC. Confocal fluorescence micrographs. Orientation: rostral to the left, dorsal to the top. Scale bar A, B, F, G 200 µm, C-E 18 µm.*

3.4 Schwann cell and NPC induced remyelination

Both, grafted Schwann cells and NPC have the capacity to remyelinate axons following spinal cord injury. Therefore, the expression of Schwann cell (P0) and oligodendroglia specific (myelin oligodendrocyte-specific protein; MOSP) myelin-associated molecules within the graft was analyzed. In both grafting paradigms containing Schwann cells (SC/FF and NPC/SC/FF), strong P0 immunoreactivity restricted to the graft site was detectable (Fig. 7A,B). The close spatial association of P0 labeling with neurofilament positive axons indicates that regenerating

axons within the graft become remyelinated by grafted Schwann cells (Fig. 7C-E). In contrast, virtually no immunoreactivity for CNS myelin was identifiable in any of the grafting conditions including NPC containing grafts (NPC/SC/FF) (Fig. 7F,G), which is not surprising knowing that the majority of co-grafted NPC leave the graft site (Fig. 2F). The only means to attribute (re-)myelination to grafted NPC is to co-localize BrdU prelabeled NPC with CNS myelin immunoreactivity. However, the antigen retrieval procedure required to detect BrdU is not compatible with immunohistochemical analysis of CNS myelin. Therefore, the degree of remyelination promoted by co-grafted NPC outside of the graft cannot be determined in the present study.

4. DISCUSSION

The aim of the present study was to investigate whether Schwann cells represent a more favorable cell population to be co-grafted with adult NPC instead of primary fibroblasts. Unlike fibroblasts, Schwann cells alone or in combination with NPC do not replace the cystic lesion defect developing following spinal cord injury. Schwann cells augment overall axonal regrowth, however, regeneration of corticospinal axons is impaired as soon as Schwann cells are introduced. Within the graft, remyelination by Schwann cells is observed, whereas almost all co-grafted NPC migrate into the adjacent spinal cord parenchyma, thus precluding remyelination through NPC derived oligodendroglia within the graft.

Adult NPC represent a promising cell-based strategy following spinal cord injury. However, adult NPC alone are not sufficient to replace typically developing cystic lesion defects [1]. In a recent study, we were able to demonstrate that fibroblasts and extracellular matrix produced by fibroblasts provide a scaffold for NPC to adhere to, thus maintaining co-grafted NPC within the lesion site [2]. Primary Schwann cells, which were highly purified (>99%) from adult sciatic nerves using MACS and consecutively introduced into co-grafts with NPC instead of fibroblasts, were not able to sufficiently replace cystic lesion defects in the present study. Once fibroblasts were added, even in low amounts, lesion replacement was almost complete. This finding is in line with previous studies, which report diminished injury induced cavitation with Schwann cell grafts containing a significant proportion of fibroblasts either by grafting less pure Schwann cell suspension grafts (95-98%) [8] or by grafting Schwann cells combined with 5% fibroblasts [18]. Apparently, highly purified Schwann cells alone do not produce sufficient extracellular matrix components qualitatively and quantitatively to replace cystic lesion defects or to serve as a platform for co-grafted NPC following transplantation into the injured spinal cord.

Another highly relevant aspect of any cell-based regenerative approach in the injured spinal cord is that grafted cells not only replace the lesion defect, but also integrate into the host spinal cord, thus building a continuous transition from the spinal

cord into the graft and back into the spinal cord. Schwann cells have been shown to promote substantial axonal regrowth into the graft, however, axons failed to reenter the distant spinal cord [19]. Schwann cells become sealed off by the surrounding spinal cord, which has been demonstrated by the upregulation of growth inhibitory extracellular matrix components such as chondroitin sulfate proteoglycans around Schwann cell grafts [20], preventing proper integration into the host. In parallel, we observed that CNS derived adult NPC and Schwann cells become separated *in vitro* and *in vivo*. Astroglial cells differentiated from NPC form glia limitans-like boundaries along streams of Schwann cells, which exactly recapitulates the segregation of astrocytes and Schwann cells in co-cultivation experiments [21]. As a consequence in vivo, the vast majority of NPC became segregated from the Schwann cell enriched graft milieu migrating into the adjacent host spinal cord. Accordingly, the absence of co-grafted NPC at the lesion site excludes significant NPC induced cell-contact mediated axon regeneration, which has contributed to the significant CST axon regrowth after co-grafting with fibroblasts [2]. Since considerable amounts of host-derived Schwann cells are shown to spontaneously migrate into the lesion site after the initial injury [14], the influence of Schwann cells on migration of already differentiated NPC needs to be investigated at long time periods after the injury.

In vitro, Schwann cells induced a differentiation shift from A2B5 expressing glial precursor cells towards GFAP expressing astroglia. The exact molecular differentiation factors responsible for this differentiation shift were not determined in the present study. Likely candidates are LIF and CNTF, which are known to be released by Schwann cells and to induce astroglial differentiation [22-25]. Since we did not observe a shift towards neuronal differentiation in NPC/Schwann cell co-culture conditions, it was not surprising to see that adult NPC as part of NPC/SC/FF co-grafts differentiated exclusively into glial cells. In line with previous findings [1, 2], NPC expressed markers resembling astroglial and oligodendroglial differentiation after transplantation. Thus, neither *in vitro* nor *in vivo* studies indicate that Schwann cells might express molecular cues, which instruct neuronal differentiation.

Schwann cell containing co-grafts induced a differential axon regrowth response.

CST axonal regrowth into Schwann cell containing co-grafts was virtually absent replicating previous findings [8, 13], in contrast to fibroblast grafts, which elicited at least minimal CST regrowth in our lesion paradigm. This is remarkable because Schwann cells are considered to have an intrinsic axon regenerative capacity, whereas fibroblasts are perceived as axon growth inhibitory serving mainly as cellular source for growth factor production following transgene transfer. Most likely the pronounced segregation of Schwann cells by glia limitans formation and chondroitin sulfate proteoglycan upregulation [20] is responsible for the poor CST regrowth into Schwann cell containing grafts. This notion is supported by an *in vitro* study,

which demonstrated a clear segregation and glial limitians formation in astrocyte/Schwann cell versus astrocyte/fibroblast co-cultures [21]. Once NPC are added to Schwann cell grafts (NPC/SC/FF) they appear to compensate for the impaired CST regeneration in SC/FF grafts up to the level of pure fibroblasts grafts. One can speculate that NPC co-grafted with Schwann cells and fibroblasts may have promoted limited cell-contact mediated axonal regrowth across the graft-host border into the graft, which is still significantly less compared to NPC/fibroblast co-grafts examined in a previous study [2]. The neurofilament density, reflecting the overall axonal regeneration was increased into Schwann cell containing co-grafts. Due to the nature of the applied lesion (dorsal column transection), transected ascending proprioceptive axons most likely accounted for the increased neurofilament density within Schwann cell containing grafts, since dorsolateral or ventral axon tracts remain intact. Possibly, these sensory projections with their neurons located in the dorsal root ganglia, as opposed to CST axons with their neurons in the motorcortex, show a robust regenerative response in a peripheral nerve like milieu such as Schwann cell grafts, which might explain the differential axon regrowth response.

Schwann cell transplants have been described to remyelinate CNS axons in a phenotypically appropriate manner [13, 26] and to restore nerve conduction of lesioned CNS axons [27]. In the investigated grafting paradigms co-grafted Schwann cells expressed P0, which is exclusively expressed in peripheral myelin, indicating robust Schwann cell promoted remyelination in the spinal cord lesion site. No relevant expression of CNS myelin could be detected within NPC containing grafts, which is obviously due to the observed migration of NPC into the adjacent host spinal cord.

Taken together, the failure to replace cystic lesion defects and the impaired regeneration of severed CST axons into Schwann cell grafts precludes the combination with NPC for a clinically relevant cell-based regenerative approach. This does not mean that pure Schwann cell grafts may not be beneficial. Schwann cells readily remyelinate CNS axons and elicit regeneration of spinal and supraspinal axons [20]. Recent studies have demonstrated that Schwann cells are even superior to olfactory ensheathing cells and elicit partial functional recovery [8]. In respect to NPC, future studies need to investigate alternative approaches such as application of natural/synthetic matrices, to maintain NPC within the lesion defect as a prerequisite to allow cell-contact mediated axon regeneration across the injury site, proper target reinnervation and ultimately functional recovery.

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