Cellular therapy after spinal cord injury using neural progenitor cells

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Chapter 5

Loss of gene expression in lentivirus- and retrovirus- transduced neural progenitor cells is correlated to migration and differentiation in the adult spinal cord

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ABSTRACT

Gene transfer into multipotent neural progenitor cells (NPC) and stem cells may provide for a cell replacement therapy and allow the delivery of therapeutic proteins into the degenerating or injured nervous system. Previously, murine leukemia virus-based retroviral vectors expressing GFP from an internal EF-1alpha promoter, and lentiviral vectors expressing GFP from a hybrid CMV/ß-actin promoter have been described to be resistant to stem cell specific gene silencing. Therefore, we investigated whether these viral vectors allow stable *in vivo* gene expression in genetically modified NPC isolated from the adult rat spinal cord. *In vitro*, NPC genetically modified to express GFP using the described retroviral vector showed strong GFP expression in undifferentiated NPC. However, *in vitro* differentiation resulted in the loss of GFP expression in 50% of cells. Grafting of BrdU prelabeled NPC to the spinal cord resulted in a loss of GFP expression in 70% and 95% of surviving NPC at 7 and 28 days post grafting, respectively. The loss in gene expression was paralleled by the differentiation of NPC into a glial phenotype. Transgene downregulation although less profound was also observed in cells modified with lentiviral vectors, whereas *in vivo* lentiviral gene transfer resulted in stable transgene expression for up to 16 months. Thus *in vivo* gene expression in genetically engineered neural progenitor cells is temporally limited and mostly restricted to undifferentiated NPC using the viral vectors tested.

1. INTRODUCTION

Gene therapy holds great potential for the treatment of several central nervous system (CNS) disorders. Delivery of therapeutic molecules by gene therapy has been shown to halt or delay the progression of neurodegenerative diseases [1-3] or to augment the regenerative capacity after CNS injury [4-9].

Different cell types including Schwann cells, fibroblasts, astrocytes and neural progenitor cells (NPC) have been used for *ex vivo* gene delivery [1, 2, 5, 10, 11]. NPC are particularly interesting due to their ability to give rise to both new neurons and glia and thus to replace lost spinal cord tissue in an organotypically appropriate manner [12, 13]. Adult NPC can be isolated from the patient's own neural tissue by minimally invasive biopsies [14, 15], propagated *in vitro* [16] and serve as autologous cell grafts in the lesioned spinal cord [17]. Cellular delivery of therapeutic genes via NPC grafts will likely require long-term transgene expression for the treatment of genetic deficiencies or chronic neurodegenerative diseases. In contrast, short-term, transient gene expression might be more desirable under certain conditions, e.g. to induce differentiation of grafted NPC into a specific phenotype that is able to enhance regeneration in the injured spinal cord [18].

To genetically modify NPC, MLV-based retroviral and lentiviral vectors have been used in several studies as these viruses allow stable integration of the transgene into the host genome [19, 20]. We and others

have previously shown that MLV-based retroviral vectors can efficiently transduce primary adult rat NPC *in vitro* [13, 21]. However, the downregulation of gene expression is a major obstacle for the persistent gene transfer into stem cells and adult NPC for long-term gene delivery [13, 21-23]. Retroviral silencing has been attributed to reduced transcriptional initiation at the promoter caused by the binding of trans-acting factors to silencer elements that are located in the viral long terminal repeats (LTRs). Furthermore, chromatin condensation caused by de novo cytosine methylation of CpG sequences located within LTRs may also contribute to transgene downregulation [22, 24]. The construction of viral vectors lacking silencer elements, the inclusion of improved positive regulatory elements [25] and the use of internal promoters have been reported to improve duration of gene expression [26]. Lentiviruses could potentially be susceptible to the same silencing mechanisms as retroviruses [27]. However, recent reports have indicated that lentiviral vectors are more resistant to stem cell specific gene silencing in various types of stem cells [28, 29]. The ability of lentiviral vectors to induce stable transgene expression in adult NPC has not been investigated to date.

In the present study we compared 3 different viral vector systems for their ability to mediate stable gene expression in NPC after engraftment into the adult spinal cord. Under these conditions NPC are known to differentiate into glial phenotypes. MLV-based retroviral vectors containing a *Xenopus* EF1a enhancer/promoter or a 5' LTR to express the reporter gene green fluorescent protein (GFP), or a lentiviral vector containing a hybrid CMV/ß-actin promoter were used to modify primary adult NPC. Two of these viral vectors have previously been described to be resistant to stem cell specific gene silencing in embryonic neural precursors [26] and mouse embryonic stem (ES) cells, respectively [28]. Our data indicate that the investigated viral vectors allow stable gene expression in undifferentiated NPC *in vitro*, whereas the vast majority of neural progenitor cells lose transgene expression after engraftment into the spinal cord. In contrast, *in vivo* lentiviral gene transfer results in long-term, stable GFP expression in the adult rat CNS.

2. MATERIALS AND METHODS

2.1 Animal subjects

Adult female Fischer 344 rats weighting 160-200 g were housed under standard laboratory conditions with 12 h light dark cycle. All experiments were carried out in accordance with institutional guidelines for animal care. Animals had *ad libitum* access to food and water throughout the study. All surgical procedures were performed under anesthesia 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.

2.2 Preparation of adult NPC

Adult NPC were isolated and cultured as described [13, 16]. Briefly, rats were deeply

anesthetized and killed by decapitation. The region of the complete cervical enlargement (spinal cord level C3 through T1) was dissected. After removal of the dura, 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 12.4 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 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 (Neurobasal medium with B27 supplement (Gibco, Karlsruhe, Germany), 2 µg/ml heparin (Sigma) 20 ng/ml recombinant human FGF-2 (R&D System, Wiesbaden, Germany), 20 ng/ml recombinant human EGF-2 (R&D System, Wiesbaden, Germany). Until the first passage, cells were grown in uncoated cell culture flasks to form neurospheres. Neurospheres were then dissociated using Acccutase (Innovative Cell Tech, San Diego, USA) and the NPC were subsequently grown as adherent monolayer cultures on poly-l-ornithin/laminin (P-Orn/Lam) coated cell culture flasks, Cell culture medium was changed twice per week. Cell cultures were passaged after reaching confluence, approximately every other week.

2.3 Production of viral vectors

The retroviral plasmid pLXSN-GFP (Fig. 1A) is based on the plasmid pLXSN (Clontech, Heidelberg, Germany) and was constructed and used to transduce NPC as described [13]. GFP expression in this plasmid is driven by the 5'LTR, and a neomycin resistance gene driven by an internal SV 40 promoter allows for the selection of transduced cells. The retroviral plasmid pCLE-GFP (Fig. 1B) is based on the plasmid pCLE (generous gift from N. Gaiano,) [26]. This plasmid contains the human cytomegalovirus enhancer-promoter fused to the Moloney murine leukemia virus LTR at the TATA box in the 5' U3 region[30] and an *Xenopus* EF-1 alpha regulatory element as internal promoter to drive transgene expression. To allow selection of cells that stably integrated the transgene, the internal ribosome entry site and the human placental alkaline phosphatase gene of pCLE were replaced by the SV40 promoter and a neomycin resistance gene. The GFP coding sequence was cloned into the multiple cloning site resulting in the plasmid pCLE-GFP.

For retrovirus production, 293T cells were grown in Iscove's Modified Dulbecco's Medium (IMDM; Pan Biotech, Aidenbach, Germany), with 10% FCS and were transfected with the retroviral plasmid (pCLE-GFP and pLXSN-GFP, respectively) and 2 packaging plasmids coding for the vesicular stomatitis virus (VSV) envelope and the retroviral gag/pol sequences. Retrovirus-containing supernatants were collected, filtered through a 0.45 µm syringe filter and stored at -80°C.

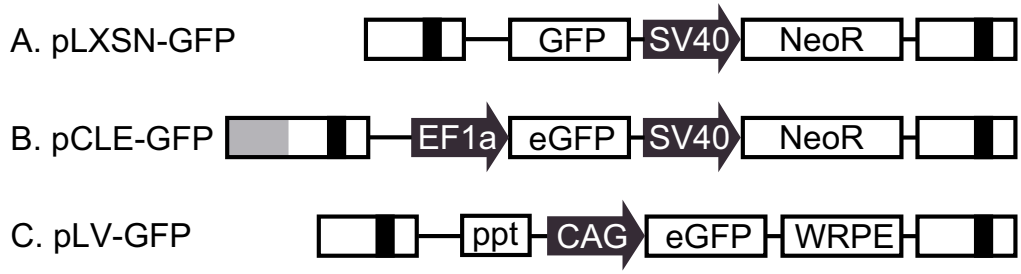

Figure 1: *Schematic representation of viral constructs. (A) In the retroviral vector pLXSN-GFP, reporter gene expression is driven by the promoter/enhancer sequences of the Moloney murine leukemia virus derived 5' LTR. (B) In contrast, the retroviral vector pCLE-GFP uses the Xenopus EF-1alpha regulatory elements (EF1a) as an internal promoter. Furthermore, pCLE-GFP contains a hybrid 5' LTR that consists of the immediate early region of the human cytomegalovirus enhancer-promoter fused to the Moloney murine leukemia virus long terminal repeat at the TATA box in the 5' U3 region. Both pLXSN-GFP and pCLE-GFP contain a neomycin resistance gene (NeoR) under control of the SV40 early promoter (SV40), which allows selection of transduced cells. (C) The lentiviral vector pLV-GFP contains the compound chicken ß-actin cytomegalovirus enhancer/ promoter (CAG) followed by a ß-globin intron for GFP expression. Furthermore, pLV-GFP contains a central polypurine tract of HIV-1 (ppt), a woodchuck hepatitis virus posttranscriptional response element (WRPE) and a self-inactivating deletion in the 3'LTR.*

For lentiviral vector production, a self-inactivating lentiviral plasmid [31] derived from pRRL [32] was used. The plasmid pLV-GFP (p156sinRRLpptCAG-GFP-PRE, Fig. 1C) containing a GFP expression cassette driven by the CMV/ß-actin hybrid promoter (CAG) [33] has been described previously [28]. This plasmid also contains a woodchuck posttranscriptional response element (WPRE) to increase gene expression [31]. For lentivirus production a third generation lentivirus packaging system was utilized as previously described [34]. Viral supernatants were concentrated by ultracentrifugation. Titers of GFP expressing virus were determined by transduction of 293T cells using serial dilutions. Viral vector stocks were also assayed for p24 antigen levels using an HIV-1 p24 specific ELISA kit (DuPont). Titers of GFP expressing virus were 5 x 10^8 IU/ml (231 µg/ml p24). For *in vitro* transduction, GFP expressing lentivirus was diluted as described below.

2.4 Transduction of NPC with retroviral and lentiviral vectors

Adult NPC were genetically modified to express the reporter gene GFP as previously described [13]. Briefly, adult NPC from passage number 4 to 6 cultures were plated in sub-confluent densities (10,000 cells/cm^2) on P-Orn/Lam coated cell culture flasks. The cells were incubated for 8 hrs on two consecutive days with retrovirus containing supernatants (pCLE-GFP or pLXSN-GFP) supplemented with 1 µg/ml Polybrene (Sigma). Approximately 60% of all cells displayed GFP fluorescence 2 days following the transduction. To select for GFP expressing cells that integrated the retroviral vector, G418 (500 µg/ml active concentration; Gibco Karlsruhe, Germany) was added to the growth medium

for 6 weeks. This concentration has previously been determined to completely eliminate all untransfected NPC within 3 weeks. Successful incorporation of the transgene into all NPC was confirmed by GFP fluorescence using an inverted fluorescence microscope (Olympus IX 70). Following G418 selection, cells were cultivated in the absence of G418.

For the transduction of NPC with lentiviral vectors (pLV-GFP), low passage NPC were split 1:3 into coated T-75 flasks (5.4 x 10^6 cells/flask). On the following day GFP expressing lentiviral vectors were added and cells were cultivated for 2 days, when GFP expression was sufficient for fluorescent activated cell sorting (FACS). Flow cytometry analysis and fluorescence microscopy indicated that 80% of the cells were positive for GFP before FACS and over 99% expressed GFP after FACS. Following FACS, cells were kept in culture and passaged twice before transplantation.

2.5 Immunocytochemistry of cultured NPC

Cells of monolayer cultures were detached using Accutase (Innovative Cell Tech, San Diego, USA) and plated on P-Orn/Lam coated glass coverslips in growth medium. To induce differentiation, NPC were incubated for up to 21 days in differentiation medium (Neurobasal medium with B27 supplement and 5% FCS, Pan Biotech, Aidenbach, Germany, [13, 35].

The following antibodies were used to analyze the differentiation pattern of adult NPC *in vitro*: rabbit-anti-GFP for GFP expressing cells (Molecular Probes, Leiden, The Netherlands; at 1/1000) mouse-anti-A2B5 for glial committed progenitors (Chemicon, Hofheim, Germany; at 1/100), 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 early neuronal differentiation (clone 5G8; Promega, Mannheim, Germany; at 1/500). Cells were fixed in 4% paraformaldehyde in PBS, washed three times with tris-buffered saline (TBS), blocked with TBS containing 3% donkey serum/0.1% Triton-X (TBS++) and incubated overnight with primary antibody in TBS++ at 4°C. To label cell surface markers, Triton-X was omitted. The following day, cells were washed with TBS and incubated with fluorescein linked secondary donkey-anti-rabbit antibodies and rhodamine-X linked secondary donkey-anti-mouse antibodies (Jackson, Hamburg, Germany; at 1/1000) in TBS++ 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).

2.6 Surgical procedures

Adult NPC virally transduced to express GFP were incubated for 48 hrs before

transplantation with a 1 µM solution of the proliferation marker bromodeoxyuridine (BrdU; Sigma) in growth medium, which results in labeling of more than 95% of all NPC. GFP expression was confirmed using an inverted fluorescence microscope (Olympus IX 70) just before harvesting the cells. An aliquot of the cells was counted in a Neubauer hemocytometer using trypan blue staining and the cells were resuspended in Dulbecco's PBS to yield a final concentration of 10^5 cells/µl.

A total of 27 animals was used in the experiments. Animals were anesthetized using a cocktail of ketamine, xylazine and acepromazine as described above and received injections of NPC transduced with the retroviral vector pCLE-GFP (n=12) or the vector pLXSN-GFP (n=3) into the cervical spinal cord. The ligament between the cervical vertebrae C3 and C4 was incised and a 2 µl cell suspension (10^5 cells/µl) was injected 1 mm deep into the spinal cord parenchyma through a stereotactically guided pulled glass micropipette (40 µm internal diameter) using a Picospritzer II (General Valve, Fairfield, USA). Animals with pCLE-GFP transduced cells were sacrificed after 2 days (n=4), 7 days (n=4) or 28 days (n=4), animals receiving pLXSN-GFP transduced NPC were sacrificed after 28 days (n=3). The lentivirus-transduced cells were grafted into the thoracic spinal cord at level T7/8 (n=3) as described above and were sacrificed after 28 days.

To control for potential unspecific labeling from unincorporated BrdU or death of BrdU prelabeled cells, a suspension of BrdU prelabeled NPC (10^5 cells/µl) was lysed by repeated freeze-thaw cycles and injected into 2 animals (2 µl per animal). Two additional animals received an injection of 2 µl PBS used to wash the cells after BrdU incubation before grafting.

For direct injection of lentiviral vectors, animals (n=5) were anesthetized as described above and received a small laminectomy at T7/8. Lentiviral vectors (2 µl) were injected 1 mm deep into the spinal cord through a pulled micropipette using a Picospritzer at a rate of 1 µl/min. Pipettes were left in place for one additional minute before withdrawal. 5 animals received lentiviral vectors containing the coding sequence for GFP and were killed at 14 days (n=3), 10 months (n=1) and 16 months (n=1).

2.7 Histological analysis

Two days, 7 days and 28 days after cell injections, and 14 days, 10 months and 16 months after lentivirus injections, animals were deeply anesthetized and transcardially perfused with 100 ml phosphate buffered saline followed by 250 ml 4% paraformaldehyde in 0.1M phosphate buffer. The brains and spinal cords were dissected, post fixed overnight and cryoprotected in 30% sucrose. Sagittal 35 µm sections were cut on a freezing microtome and processed for immunohistochemistry. Every seventh section was immediately mounted and Nissl stained to localize the injection site.

For brightfield immunohistochemical analysis of BrdU prelabeled cells, sections were rinsed in TBS (0.1M) and incubated

for 1h in 50% formamide/2xSSC (0.3 M NaCl, 0.03M sodium citrate) at 65°C. Sections then were rinsed in 2xSSC, incubated for 30 min in 2 N HCl at 37°C, and rinsed for 10 min in 0.1 M boric acid pH 8.5. After rinsing in TBS, sections were incubated in TBS++ for 1h, transferred into rat-anti-BrdU primary antibody (Harlan SeraLab, Loughborough, UK; at 1/500) in TBS++ and incubated overnight at 4°C on a rotating platform. On the second day, the sections were washed in TBS and incubated for 1h with a biotinylated donkey-anti-rat secondary antibody (Jackson, Hamburg, Germany; at 1/2000) in TBS++. After several rinses in TBS, the sections were incubated for 1h with avidin biotinylated peroxidase complex (Vector Elite Kit, Vector Laboratories, Wertheim, Germany; at 1/1000). Labeling was developed using a 0.05% solution of 3,3,8-diaminobenzidine, 0.01% H_2O_2, and 0.04% nickel chloride in TBS for 5 min. Sections were mounted, dehydrated and coverslipped using Neo Mount (Merck, Darmstadt, Germany). The brightfield immunohistochemical analysis was performed with a Leica DMR microscope equipped with a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Michigan, USA).

Double/triple immunofluorescence labeling was performed to assess GFP expression of NPC and lentivirus transduced cells in the spinal cord, and the differentiation pattern of grafted adult NPC. The following primary antibodies were used: mouse-anti-nestin for uncommitted progenitor cells (clone 401, Pharmingen, Heidelberg, Germany; at 1/500), guinea pig-anti-NG2 for glial restricted precursor cells (; generous gift B. Stallcup, Burnham Institute, La Jolla, USA; at 1/200), mouse-anti-GFAP for astroglia (Chemicon, Hofheim, Germany; at 1/600), mouse-anti-APC that is directed against the N-terminal segment of APC for immature and mature astrocytes and oligodendrocytes [36] (Oncogene, Darmstadt, Germany; at 1/1000) and goat-anti-doublecortin for young neurons (Santa Cruz, Heidelberg, Germany; at 1/100), all visualized using Cy-5 linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000). The BrdU-prelabeled grafted cells were identified with a rat-anti-BrdU antibody (Harlan SeraLab, Loughborough, UK; at 1/500) and visualized using Rhodamine-X linked donkey secondary antibodies (Jackson, Hamburg, Germany; at 1/1000). GFP expressing cells were visualized using a rabbit-anti-GFP antibody (Molecular Probes, Leiden, Netherlands; at 1/750) or a goat-anti-GFP antibody (Rockland Gilbertsville, PA; at 1/1500).

To identify BrdU prelabeled NPC sections were pretreated as described above followed by incubation with the primary antibodies in TBS++ overnight at 4°C on a rotating platform. The following day, sections were rinsed and incubated with fluorescence (rhodamine-X, fluorescein, Cy5) conjugated secondary antibodies made in donkey (Jackson, Hamburg, Germany; at 1/1000) in TBS++ for 2 h. After final rinsing steps in TBS, sections were mounted onto glass slides and coverslipped with ProLong Antifade Kit (Molecular Probes, Leiden, Netherlands). Immunohistochem-

ical analysis was performed with a confocal fluorescence microscope (Leica TCS-NT).

For the quantification of BrdU/GFP double-labeled cells, confocal micrographs containing the epicenter of the graft were taken at 40x magnification. From each animal, 3 independent image stacks were selected from different sections. Since GFP labeling in NPC was restricted to the injection site, cells were only quantified at the transplantation site. Therefore, between 15-20 optical sections through the z-axis of the 35 µm thick section were analyzed. Co-localization was confirmed once the GFP marker was spatially associated to the nuclear BrdU prelabeling through subsequent optical sections in the z-axis. BrdU positive cells and GFP/BrdU positive cells were counted from each animal to obtain a representative total count. Finally, the mean percentage of BrdU/GFP double labeled cells and the standard error of the mean (SEM) was calculated for each time point. Group comparisons were made using an analysis of variance followed by Tukey's post-hoc testing.

3. RESULTS

3.1 In vitro gene expression using the retroviral vector pCLE-GFP

Previously, we have shown that neural stem cells derived from the adult spinal cord can be transduced *in vitro* to express GFP using the retroviral vector pLXSN-GFP [13] (Fig. 1A). However, *in vivo* gene expression was lost in the majority of cells 21 days after transplantation into the adult injured spinal cord [13]. Because this loss in gene expression is likely due to the silencing of the LTR used to express the transgene, we investigated whether retroviral vectors with an internal promoter could increase the duration of gene expression. The retroviral vector pCLE-GFP derived from pCLE [26] contains a hybrid 5' LTR and an internal *Xenopus* EF1a enhancer/promoter to drive GFP expression (Fig. 1B). Adult spinal cord derived NPC were efficiently transduced and showed robust GFP expression after selection for G418 resistance. GFP expression was stable for at least 4 weeks over more than 5 passages *in vitro* after removal of G418 from the cell culture medium (Fig. 2A). To determine if differentiation of NPC results in a downregulation of transgene expression [37], NPC were cultivated in differentiation medium (NB medium, supplemented with B27 and 5% FCS; no G418) for up to 21 days [13, 35]. After 7 days of incubation in the differentiation medium, 99.2% ± 0.48 of the cells were GFP positive (Fig. 2B, 2I). Immunocytochemical staining showed that these cultures mainly contained beta III tubulin positive neuronal cells (13.4% ± 3.6, Fig. 2F, 2J) and GFAP positive astrocytes (31.3% ± 1.8, Fig. 2G, 2K). Only a small amount of GalC positive oligodendrocytes and A2B5 positive glial precursor cells could be identified (Fig. 2E, H). After 14 days of incubation in the differentiation medium, however, a considerable proportion of the pCLE-GFP transduced

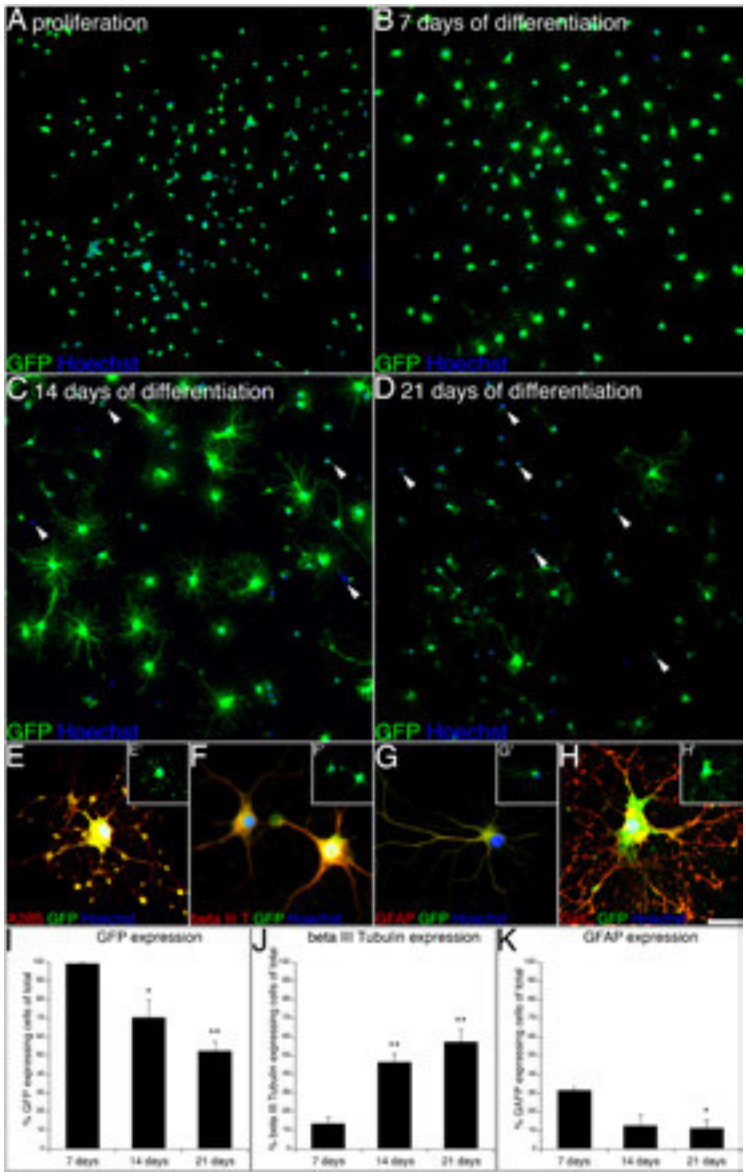

Figure 2: *In vitro analysis of NPC transduced with pCLE-GFP. (A) Under proliferation conditions and (B) after 7 days of cell culture under differentiation conditions virtually all NPC transduced with the retroviral vector pCLE-GFP expressed the reporter gene GFP (green). (C) After 14 days and (D) after 21 days of cell culture under differentiation conditions, many pCLE-GFP transduced NPC lost GFP expression (arrowheads). (E) Immunocytochemical analysis of the differentiated cells showed that A2B5 positive glial restricted progenitor cells, (F) beta III tubulin positive young neuronal cells, (G) GFAP positive astrocytes and (H) GalC positive oligodendrocytes could be found to express GFP (green) in vitro after 7 days of in vitro differentiation. All differentiation markers are shown in red. Hoechst 33342 was used as nuclear counterstain (blue). Epifluorescent micrographs, scale bar: 75 µm in (A-D), 20 µm in (E-H). (I) Bar graphs showing the proportion of GFP expressing cells, (J) beta III tubulin expressing young neurons and (K) GFAP expressing astrocytes of the total amount of cells present in the culture after 7, 14 and 21 days of in vitro differentiation (n=3, mean ± SEM, * p<0.05, ** p<0.01).*

NPC lost GFP expression, resulting in a significant reduction in GFP-labeled cells to 70.2% ± 9.4 ($p<0.05$; Fig. 2C, 2I). After 21 days of *in vitro* differentiation, the proportion of pCLE-GFP transduced NPC that still expressed GFP was further reduced to 52.4% ± 5.4 of the total amount of plated cells ($p<0.01$; Fig. 2D, 2I). The amount of beta III tubulin positive cells increased to 46.3% ± 4.5 at 14 days and to 57.2% ± 6.6 at 21 days of *in vitro* differentiation (Fig. 2J), whereas the amount of GFAP positive cells decreased to 12.4% ± 6.0 at 14 days and 11.0% ± 4.7 at 21 days (Fig. 2K). Only very few GalC positive oligodendrocytes and no A2B5 positive glial precursor cells could be identified at 14 and 21

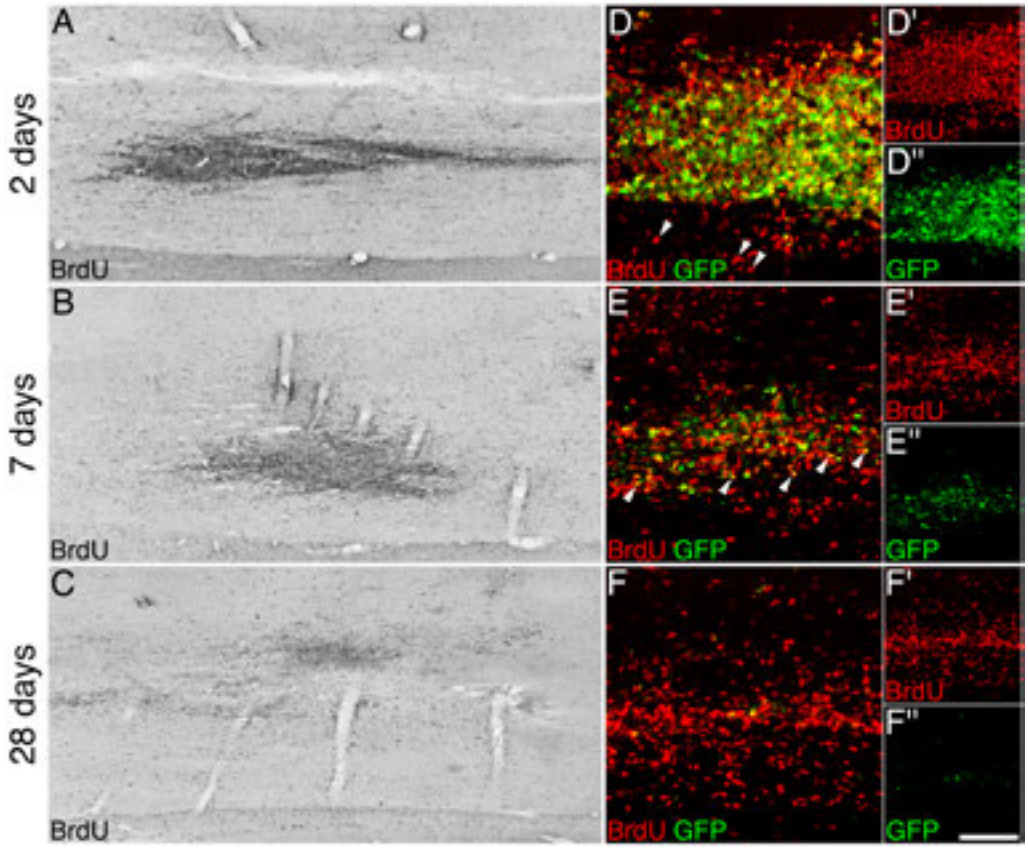

Figure 3: *Genetically modified NPC migrate in the spinal cord and lose transgene expression. (A) 2 days post-transplantation, the majority of the BrdU prelabeled NPC were concentrated in a cloud of cells at the injection site. (B) 7 days and (C) 28 days post-transplantation, many of the grafted cells migrated into the host parenchyma and were scattered in the host spinal cord. (D) Two days post-transplantation, GFP (green) could be detected in most of the BrdU prelabeled NPC (red) directly at the injection site. Cells that migrated away from the injection site had already downregulated GFP expression (arrowheads). (E) At 7 days post-grafting, the number of GFP expressing cells was further diminished, while many other cells expressed only very low levels of GFP (arrowheads, compare also D'' with E''). (F) 28 days post-transplantation, GFP labeling had nearly disappeared. Scale bar: 250 µm in (A-C), 60 µm in (D), 80 µm in (E, F).*

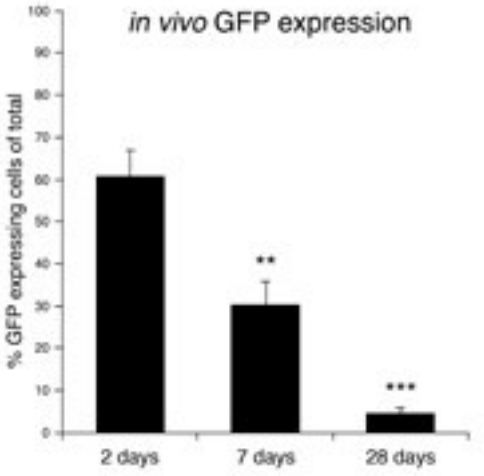

Figure 4: *Quantification of BrdU/GFP double-labeled cells in vivo. Bar graph showing the proportion of BrdU-labeled NPC that colocalize with GFP at the injection site. There is a significant reduction in the number of GFP-expressing cells over time. (n=3/time point, mean± SEM, ** p<0.01, *** p<0.001).*

days of *in vitro* differentiation. Because the proliferation of NPC ceases over the first 7 days of cultivation in differentiation medium, the observed decline in GFP expression was not due to the proliferation of non-transduced cells. Rather the percentage of GFP-labeled cells decreased over time as the proportion of cells expressing neuronal and glial differentiation markers increased.

3.2 In vivo gene expression in NPC grafted to the spinal cord

To investigate whether a similar degree and time course of transgene silencing would also occur *in vivo*, undifferentiated, pCLE-GFP-transduced NPC were labeled with BrdU and grafted to the intact spinal cord of adult rats. At 2 days, 7 days and 28 days post grafting, the spinal cord parenchyma of grafted animals contained many BrdU positive nuclei, indicating good survival of transplanted NPC (Fig. 3A-C). Two days post grafting, the majority of BrdU positive nuclei were localized in a compact cloud of cells at the transplantation site (Fig. 3A). Surrounding the graft epicenter, many BrdU positive nuclei resided in the spinal cord parenchyma at a lower density, suggesting that a fraction of the grafted cells had already migrated into the host parenchyma. At 7 days and 28 days after transplantation, the density of BrdU prelabeled nuclei at the graft epicenter decreased, and the majority of cells had migrated away from the transplantation site (Fig. 3B, C). Control animals injected with BrdU prelabeled NPC lysed by repeated freeze-thaw cycles prior to transplantation, or PBS used to wash the cells before grafting contained none or occasionally very few BrdU labeled nuclei. Thus, the BrdU positive cells in the spinal cord parenchyma of grafted animals originated from surviving, transplanted NPC.

Concomitant with the migration of NPC, expression of GFP was gradually lost (Fig. 3D-F). Two days post grafting, many BrdU/GFP double-labeled cells were detected at the graft epicenter (Fig. 3D). However, confocal analysis of BrdU positive cells residing outside the graft epicenter revealed that these cells almost completely downregulated GFP expression (Arrowheads, Fig. 3D). Quantification of BrdU/GFP double-labeled cells at the graft epicenter indicated that 60.8% ± 6.0 of the BrdU positive nuclei co-localized with the GFP reporter (Fig. 4). At 7 days

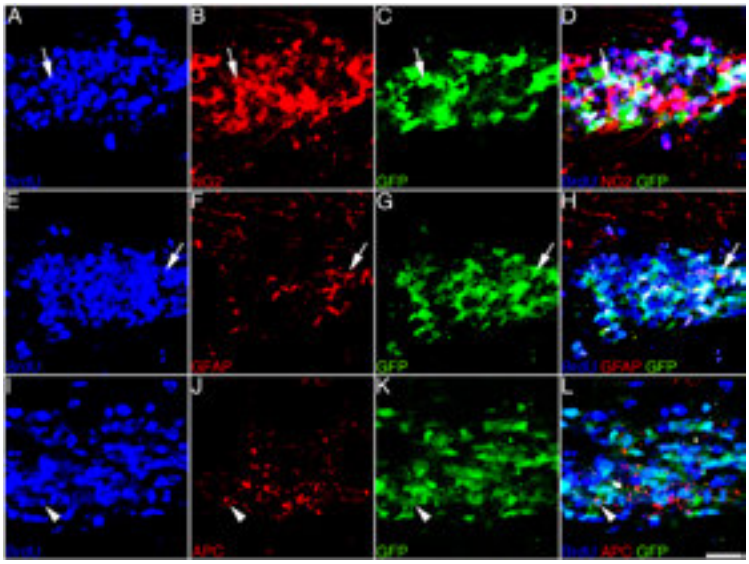

Figure 5: *GFP expression and differentiation of pCLE-GFP transduced NPC at 2 days after transplantation into the spinal cord. The differentiation pattern of grafted NPC was assessed by determining the co-localization of differentiation markers (red) with BrdU prelabeled nuclei of grafted cells (blue). (A-D) 2 days after transplantation, many grafted cells expressed the glial precursor cell marker NG2 (arrow). (E-H) Only very few grafted cells could be detected co-localizing with the astroglial marker GFAP (arrows) and (I-L) the mature glial marker APC (arrowhead). Only very few GFP expressing cells (green) could be found that co-localized with GFAP or APC (A-H, arrows). Confocal fluorescence micrographs, scale bar A-L 25 µm.*

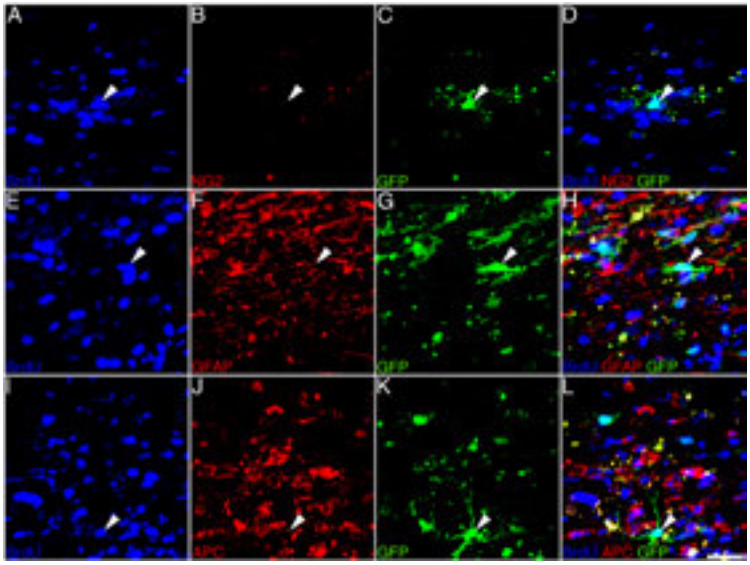

Figure 6: *GFP expression and differentiation of pCLE-GFP transduced NPC at 28 days after transplantation into the spinal cord. (A-D) At 28 days post grafting, no NG2 immunoreactivity could be detected that co-localized with the grafted NPC. However, most grafted cells differentiated into glial phenotypes, as indicated by (E-H) GFAP and (I-L) APC expression. Although many GFP labeled NPC displayed a complex morphology, almost none of these cells could be co-localized with the investigated phenotypical markers (arrowheads, D, H, L) Confocal fluorescence micrographs, scale bar A-L 25 µm.*

post grafting, the fraction of BrdU/GFP double labeled cells at the graft epicenter decreased to 30.3% ± 5.6 (Fig. 3E, Fig. 4) and many of the remaining BrdU/GFP double positive cells displayed only weak GFP expression levels (arrowheads, Fig. 3E). At 28 days post grafting, only 4.3% ± 1.5 BrdU/GFP double positive cells could be identified at the graft epicenter, indicating that the vast majority of pCLE-GFP-transduced NPC lost expression of the GFP reporter gene within 28 days after transplantation into the intact CNS (Fig. 3F, 4). Double-labeled cells were only quantified at the injection site, since cells migrating for long distances were never found to express GFP. Thus, the actual proportion of GFP/BrdU positive cells in the whole spinal cord was even lower than quantified in this study, especially at 7 and 28 days post-injection, when long distance migration of BrdU prelabeled cells could be observed.

To determine if the loss in gene expression was correlated to differentiation of NPC, colocalization studies of BrdU- and GFP-labeled cells with NG2, a marker for glial committed precursor cells [38] and the glial markers GFAP and APC were conducted. At 2 days post grafting, BrdU prelabeled pCLE-GFP-transduced NPC mainly expressed NG2 (Fig. 5A-D), few cells expressed GFAP (5E-H), and no BrdU-labeled cells co-labeled with APC (Fig. 5I-L). At 28 days post grafting, NG2 immunoreactivity had almost completely disappeared, while many of the grafted pCLE-GFP-transduced NPC differentiated into GFAP positive astrocytes (Fig. 6E-H) and APC positive glia (Fig. 6I-J). No immunoreactivity for the multipotent progenitor cell marker nestin[39] or for the early neuronal marker doublecortin [40] could be detected at any of the time-points investigated (data not shown). Strikingly, only a very small fraction of GFP/BrdU double-labeled cells could be co-localized with any of the glial or neuronal markers investigated (Arrows, Fig. 5A-D, E-H), suggesting that the vast majority of transduced NPC downregulated GFP expression upon or during differentiation. The few NPC that continued to express GFP at 28 days post transplantation displayed a highly complex morphology without detectable levels of any of the investigated differentiation markers (Arrowheads, Fig. 6D, H, L).

3.3 Lentiviral gene transfer

The studies described above indicated that the differentiation of NPC might be responsible for the discontinued gene expression in NPC transduced with MLV-based retroviral vectors. As lentiviral gene transfer into embryonic stem cells [28 41] and hematopoetic stem cells [42] has been shown to be stable after differentiation we tested the stability/duration of lentiviral gene expression in NPC. In parallel to findings with the retroviral vectors pLXSN-GFP and pCLE-GFP (see above), NPC transduced with the lentiviral vector pLV-GFP (Fig. 1C) showed stable *in vitro* GFP expression in proliferating cells (data not shown). Upon transplantation of pLV-GFP transduced NPC into the spinal cord however, gene expression declined

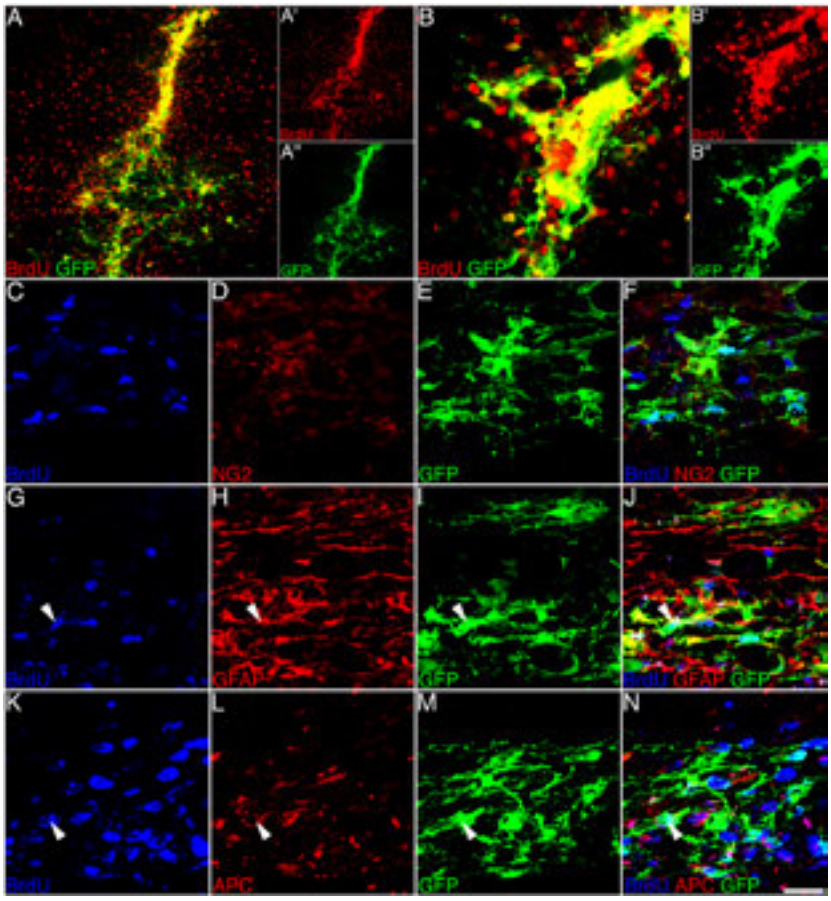

Figure 7: *GFP expression and differentiation of pLV-GFP transduced NPC at 28 days after transplantation into the spinal cord. (A, B) Although a larger proportion of lentivirus-transduced NPC continued to express GFP compared to MLV-retrovirus-transduced NPC, the majority of the grafted cells lost transgene expression 28 days after transplantation. (C-F) Similar to pCLE-GFP transduced NPC, no NG2 expressing grafted NPC could be found at 28 days post grafting, while most NPC differentiated into (G-J) GFAP expressing astroglia and (K-N) APC expressing mature glia. Only few GFP-labeled NPC could be colocalized with the investigated phenotypical marker (arrowheads, G-N). Confocal fluorescence micrographs, scale bar A 170 µm, B 32 µm, C-N 25 µm.*

to 29.2% ± 4.7 at 28 days post grafting around the injection site (Fig. 7A, B). As previously observed for pCLE-GFP transduced NPC, cells remaining close to the injection site were more likely to continue GFP expression than cells that migrated away from the transplantation site (Fig. 7A). Confocal analysis of grafted cells showed that almost no colocalization with the glial precursor cell marker NG2 could be observed at 28 days (Fig. 7C-F). Of the investigated glial differentiation markers, only a small fraction of GFP/BrdU-labeled cells colocalized with GFAP (arrowheads, Fig. 7G-J) or APC (arrowheads, Fig. 7K-N). To determine if long-term GFP expression could be achieved in differentiated cells*in vivo*, and to exclude the possibility that im

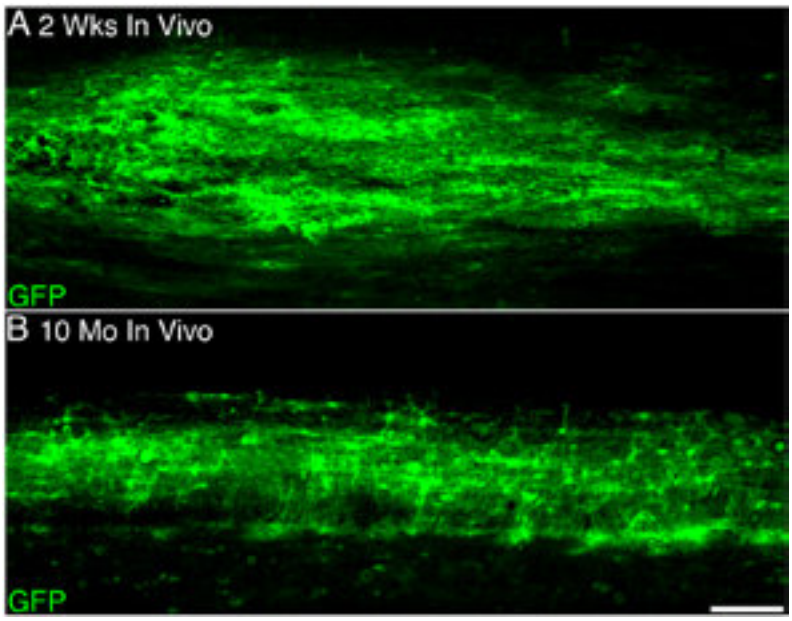

Figure 8: *GFP expression after direct injection of pLV-GFP virus into the intact rat spinal cord. (A) Direct injection of lentivirus resulted in high expression levels of transgene in the intact rat spinal cord 2 weeks post-injection. (B) No obvious changes in transgene expression level or number of expressing cells could be observed at 10 months post-injection. Epifluorescence micrographs, scale bar A, B 200 µm.*

mune responses to GFP expressing cells provided a selective survival advantage for cells that lost GFP expression, we injected the same lentiviral vectors expressing GFP into the spinal cord. Qualitative analysis of gene expression 14 days, 10 months and 16 months after virus injection indicated continued gene expression without any appreciable loss in the amount or number of GFP expressing cells (Fig. 8).

4. DISCUSSION

In the present study, we investigated transgene expression in adult rat NPC transduced to express GFP using retroviral and lentiviral vectors that have previously been described to be resistant to gene silencing in neural progenitor cells and stem cells [26, 28]. Our findings indicate that the viral vectors tested only allow for transient gene expression in adult NPC. *In vitro* differentiation of NPC and *in vivo* differentiation following transplantation to the adult spinal cord was paralleled by a rapid loss of GFP expression indicating a close relationship between NPC differentiation and transgene silencing. In contrast, direct *in vivo* injection of lentiviral vectors, resulted in stable GFP expression in both neurons and glia for up to 16 months. The stable expression of transgenes in adult NPC represents an important prerequisite to assess and to enhance the regenerative capacity of NPC grafting to the CNS. The introduction of reporter genes such as GFP enables the visualization of grafted cells to study cell survival, integration and interaction with the host en-

vironment. Additionally, overexpression of specific genes could allow the differentiation of grafted NPC into regeneration promoting phenotypes thereby overcoming differentiation inducing cues in the microenvironment at the transplantation site [35, 43]. Finally, the intrinsic regenerative capacity of grafted NPC could be enhanced by overexpression of axonal growth and neuronal survival promoting factors [44, 45].

In vitro and *in vivo* loss of gene expression in our experiments could be due to the toxicity of GFP expression, selective survival advantages of cells that lose gene expression, elimination of grafted cells, or silencing of transgene expression previously described for many virally transduced cell types [46-48]. The first three possibilities seem highly unlikely for several reasons: 1) NPC cultivated *in vitro* under proliferation conditions continued to express GFP without any sign of toxicity or loss of gene expression; ; 2) direct injection of GFP expressing lentiviral vectors resulted in long-term stable gene expression for up to 16 months; 3) grafted cells could be identified in all animals using BrdU pre-labeling and 4) a decline in the number of GFP expressing cells was also observed *in vitro* in differentiating NPC. Although the exact molecular basis of transgene silencing remains to be elucidated, DNA-methylation-dependent and -independent silencing mechanisms appear to play important roles [49]. Previous studies have shown that transgene silencing in stem/progenitor cells precedes methylation of newly integrated virus DNA. Whether this de novo methylation is a consequence of methylation-independent silencing or represents a redundant parallel pathway is unclear [27]. The dramatic gene silencing within 1 week post-grafting in the present experiments suggests that silencing factors other than the relatively slow acting DNA methylation play a significant role. Nevertheless, the development of viral vectors lacking CpG dinucleotides located in the LTR represents an important step in preventing methylation-dependent transgene silencing [24]. Inclusion of insulator elements, which are genomic sequences that function as expression boundaries, might also be able to reduce methylation-independent transgene silencing [25]. However, it remains to be determined whether insulators are also able to block the function of trans-acting retroviral silencers [50, 51].

Two of the viral vectors used in our studies have previously been described to be more resistant to stem cell specific transgene silencing compared to retroviral vectors expressing the transgene from the 5'LTR. The retroviral vector, pCLE-GFP, contains a hybrid 5' LTR in combination with an internal *Xenopus* EF-1alpha enhancer/promoter and has been shown to improve transgene expression after injection into the embryonic mouse CNS [26]. Our results did not indicate significant improvements in transgene expression using this viral vector. Differences between mouse and rat CNS progenitors, differences between adult and embryonic silencing mechanisms, or differences in the multiplicity of infection might have contributed to the observed lack of extended

gene expression.

The tested lentiviral vector pLV-GFP reported to be resistant to stem cell specific silencing in murine ES cells[28] only resulted in a slightly higher number of transgene expressing NPC close to the injection site. Lack of gene silencing in lentivirus-transduced ES cells has been attributed to the deletion of enhancer/promoter regions in the LTR regions of the virus construct and intrinsic resistance to long-term DNA methylation-dependent silencing of human derived lentiviral vectors in murine cells [28]. Whether this viral vector is sufficient to induce stable transgene expression in rat stem cells has not been investigated. The observed differences could therefore be due to cell-specific or species-specific differences. Recent studies in lentivirus-transduced embryonic rat NPC using an internal mouse phosphoglycerate kinase 1 promoter have observed a similar decline in gene expression [52].

Consistent with previous reports [13, 17], NPC grafted to the spinal cord differentiated rapidly into glial lineages. NG2 expressing BrdU-labeled cells however could only be detected at 2 days post-injection. Cells that continued to express GFP 7 and 28 days post-grafting typically resided close to the graft epicenter, similar to retrovirus-transduced NPC grafted to the striatum of hemiparkinsonian rats [23]. Strikingly, a lack of GFP expression in cells that migrated away from the injection site could be observed as early as 2 days post transplantation, indicating that migration of the grafted cells closely correlates with the silencing of transgene expression. Although some GFP expressing cells displayed highly complex morphologies at 4 weeks post transplantation, only relatively few GFP expressing cells could reliably be co-localized with the tested differentiation markers, confirming earlier observations [23].

In summary, the viral vectors investigated in the present experiments can only be employed to induce transient gene expression in rat NPC. Studies using viral vectors to introduce transgenes into NPC have to be carefully controlled for transgene expression levels throughout the entire experiment as only a specific subset of cells might continue to express the gene of interest. To accomplish stable transgene expression in adult rat NPC, viral vectors need to be developed that are both resistant to methylation-dependent and -independent silencing mechanisms. Viral vectors that can also persist without integration into the host cell genome, such as adeno-associated viruses, might be able to at least partially overcome stem cell specific silencing in stem/progenitor cells [53]. Whereas expression of reporter genes, survival promoting factors in chronic neurodegenerative diseases, and genes to correct genetic deficiencies are generally sought to be long-lasting, a loss of gene expression that is correlated to cell differentiation as observed in the current experiments might be ideal to express genes necessary for the phenotypical specification of stem and progenitor cells.

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