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RESEARCH ARTICLE

Transduction of human neural progenitor cells using recombinant adeno-associated viral vectors

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Human neural progenitor cells (hNPCs) represent an attractive source for cell therapy of neurological disorders. Genetic modification of hNPCs may allow a controlled release of therapeutic proteins, suppress immune rejection, or produce essential neurotransmitters. In search of an effective gene delivery vehicle, we evaluated the efficiency of a recombinant adeno-associated viral (rAAV) vector expressing enhanced green fluorescent protein (CAGegfp). Our study demonstrated that CAGegfp efficiently transduced both proliferating and differentiated hNPCs in vitro. EGFP expression was detected as early as 1 day after exposure to CAGegfp and was detectable for up to 4 months. Following transduction, the growth rate of hNPCs slowed down, but they were still able to differentiate into neurons and glia. Furthermore, CAGegfp-modified hNPCs survived, differentiated and expressed EGFP after transplanting into spinal cord of adult rats. Our results indicated that rAAV vectors might be a useful tool in hNPC-based cell and gene therapy for neurological disorders.
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Keywords: *human neural progenitor cells; adeno-associated virus; green fluorescent protein; rat; spinal cord; transplantation*

Introduction

It is possible that future approaches to treating neurological diseases may include the transplantation of cells to either produce therapeutic proteins, or replace lost cells.[1] This is based on studies using fetal tissue transplants from both animals and humans, which are able to survive, differentiate and incorporate into the adult host environment.[2–5] In some cases, neural transplants were able to promote functional recovery in animals with experimentally induced damage to the nervous system.[6–8] However, fetal tissues are difficult to obtain. Furthermore, possible contaminations and heterogeneity of donors may render such transplantation impractical as a widespread therapy.[9–11] Recent studies using immortalized neural progenitors have shown they can be both extensively expanded in culture, and survive transplantation into the intact or damaged CNS.[9,12–14] However, immortalization carries with it the risk of tumor formation following grafting, and may not be the optimum choice for clinical applications.[15,16]

Neural stem cells (NSCs), defined by their ability to self-renew over many generations while retaining the potential to generate both neurons and glia, are another potential source of tissue for cell therapy in the CNS.[15,16] They are not immortalized, but can be expanded as spherical aggregates, or 'neurospheres', to a large number of cells required for replacing damaged neurons.[17] Neurospheres contain some NSCs, but the majority of cells within these aggregates are more restricted, but mitotically active, neural progenitor cells (hNPCs).[18] Similar cells have recently been isolated from either embryonic,[19–22] or adult human brain tissues,[23–25] although the exact relationship to hNPCs derived from embryonic tissues remains to be established. Given the human origin, capability to differentiate into neurons and incorporate into host CNS,[22,26,27] hNPCs have attracted a great deal of attention as a promising donor source for xenographic or allographic transplantation.

hNPCs may also provide a unique source of cells for *ex vivo* gene therapy approaches. The first step toward this goal is to develop a system that will deliver foreign genes into these cells in an efficient, stable, regulatable and safe manner. It has been reported previously that adenovirus vectors (AV) efficiently transduced primarily cultured human neural progenitor cells.[28,29] However, strong immune response and transient gene expression related to AV vectors make them inappropriate for most clinical usages to treat neurological disorders. More recent studies have demonstrated that primary or immortalized hNPCs could be transduced with retroviral vectors and expressed foreign genes.[26,30] However, the retrovirus requires cell division to integrate, making infection of differentiated postmitotic neural cells derived from hNPCs impossible. This may be desirable to modify the cells following their selective differentiation into either neurons or glia. In searching for a suitable gene delivery vehicle for both proliferating and differentiated hNPCs, we selected type II adeno-associated virus-based vector (AAV2 or AAV in this report). As one of the most promising vectors, AAV can transduce both dividing and non-dividing cells in a highly efficient and stable manner without inducing cytotoxicity or cellular immune

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response.[31–34] In addition, no link has been found between wild-type (wt) AAV and any human disease, which reduces the potential risk to human subjects. As one of the smallest human viruses, AAV contains a linear, single-stranded DNA inside its protein capsid. The genome, 4680 nucleotides in length, encodes the viral regulatory (*rep*) and structural (*cap*) proteins.[35,36] Most of the current AAV-based vectors exclude all the viral genes, and contain only two fragments of AAV DNA, termed inverted terminal repeat (ITR). These ITRs, 145-bp each, are the only essential AAV sequences required for replication, integration and packaging.[36] Previously, we demonstrated that AAV vectors could efficiently transduce and integrate into brain neurons *in vitro* and *in vivo*.[37,38] In the present study, we evaluated the capability of AAV vectors to deliver a reporter gene into hNPC *in vitro*, as well as the transgene expression in hNPC transplant in rat spinal cord *in vivo*.

Results

pCAGegfp recombinant AAV plasmid

EGFP was selected as a reporter gene in this study based on the convenience to monitor its expression in both live and fixed cells *in vitro*, as well as grafted cells *in vivo*. To ensure a strong and constitutive transgene expression,[39] we engineered a pCAGegfp plasmid by placing the EGFP gene under the control of a CAG promoter. This chimeric promoter consists of a cytomegalovirus (CMV) immediate–early enhancer, a chick β-actin promoter, a chimeric intron, exon 1 and a part of exon 2 of a rabbit β-globin gene. This hybrid promoter has a higher activity than the CMV promoter especially in mammalian cells.[39,40]

Efficient and stable transduction of dividing hNPCs with rAAV

The CAGegfp vector was used to determine the efficiency and stability of rAAV-mediated gene transfer in hNPCs. Cells expressing the marker gene turned green under an epifluorescent microscope with a FITC filter, which allowed us to continuously monitor the transgene expression in live cells. EGFP expression was detectable as early as 1 day after treatment with a high MOI (5×10^3). It is well known that the MOI determined on the total number of viral genome is equivalent to an MOI of approximately 2- to 3-log less in terms of infectious viral particles, since many defected viral particles during viral preparation are not infectious. In other words, the MOI, such as 5×10^3 determined by dot blot analysis, is about 50 based on the infectious functionality. The intensity of transgene expression increased over time and reached its peak around 2 weeks (Figure 1b), a common phenomenon noticed in rAAV transduction in many types of mammalian cells. The dosage of the vector used in Figure 1b–d was at an MOI of 2.5×10^4 based on a dot blot analysis. In addition, the rAAV-mediated EGFP expression was seen for at least 3 months *in vitro* – the latest time-point tested (Figure 1d).

To optimize transduction of the hNPCs, the dose–response study was carried out by treating medium-sized neurospheres (about 10^4 cells per sphere) with increasing amounts of CAGegfp at MOIs ranging from 5×10^1 to 5×10^3. At various time intervals (3 days, 2 weeks, 1 and 2 months), spheres were trypsinized and dispersed into single cells. The numbers of bright green fluorescent cells detected under a fluorescent lens and filter, as well as the total number of cells under a phase contrast plain lens were immediately counted manually in each of 10 randomly chosen fields per treatment (at least over 200 cells in total) under a Nikon (Lewisville, TX, USA) epifluorescent microscope. Using a FITC filter with a narrow range of excitation and emission (Chroma, Brattleboro, VT, USA), we were able to compare and distinguish readily the green fluorescent cells from cells without labeling in the same field. Experiments were repeated three times for each dosage and each time-point. Percentages of green cells were averaged over 10 fields and then over three experiments. The final mean percentages of cells with green labeling are shown in Figure 2, which indicated that higher dosages of CAGegfp vectors induced more hNPCs to express EGFP transgene. In addition, we have also observed that the hNPC spheres with a smaller size had higher transduction efficiency when exposed to the same number of AAV vectors (data not shown).

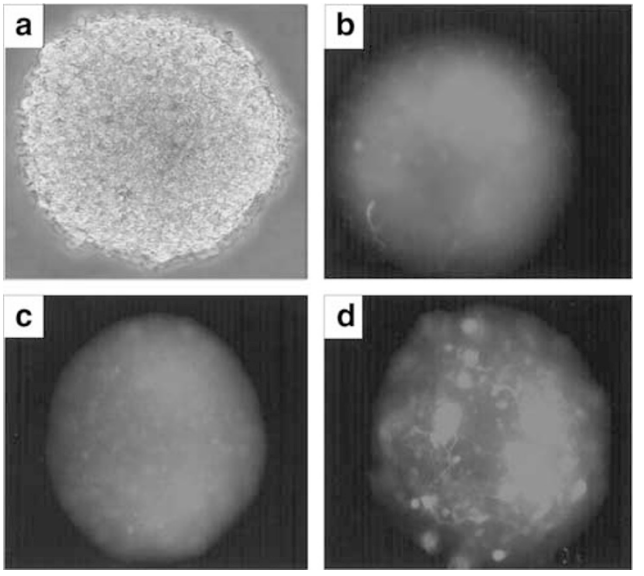

Figure 1 *Live images of hNPC neurospheres without (a) and with (b–d) rAAV transduction. (a) A phase contrast image of hNPC sphere at 25-weeks culture in vitro. (b–d) The expression of EGFP in CAGegfp-transduced hNPCs. Live images of fluorescent hNPC spheres were readily detected at 2 weeks (b), 1 month (c) and 3 months (d) after exposure to CAGegfp at an MOI of 2.5×10^4. Spheres were maintained in a growth medium containing 20 ng/ml EGF and 20 ng/ml bFGF. Note, spontaneous differentiation was occurring especially at 3 months after transduction (d) by showing processes in spheres.*

rAAV transduction decreases hNPC proliferation

In repeated experiments by transducing neurospheres with AAV vectors, we observed that CAGegfp-treated neurospheres grew slower than those untreated. Particularly, spheres with the highest transduction efficiency showed the most intensive green labeling yet seemed to cease proliferation. In addition, the intensity of green fluorescent labeling in those spheres with less transduction efficiency reduced over time, while spheres continuously grew bigger. We therefore suspected that CAGegfp treatment might affect hNPC proliferation. To further address this issue without mingling with the different efficiency of transduction due to different sizes of neu-

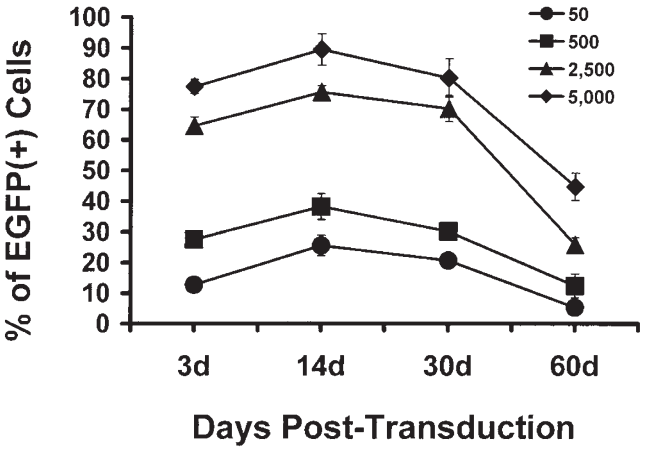

Figure 2 Quantitative dose–response and time-course studies of CAGegfp-transduced hNPCs. hNPCs were exposed to increasing dosages of CAGegfp. At various time-points, cells were dissociated and counted under a fluorescent microscope using a FITC filter. ◆, cells treated with CAGegfp at an MOI of 5×10^3; ▲, an MOI of 2.5×10^3; ■, an MOI of 5×10^2; ●, an MOI of 50. Data represent the means ± s.e. from 10 randomly chosen fields in each sample, and averaged over three repeated experiments.

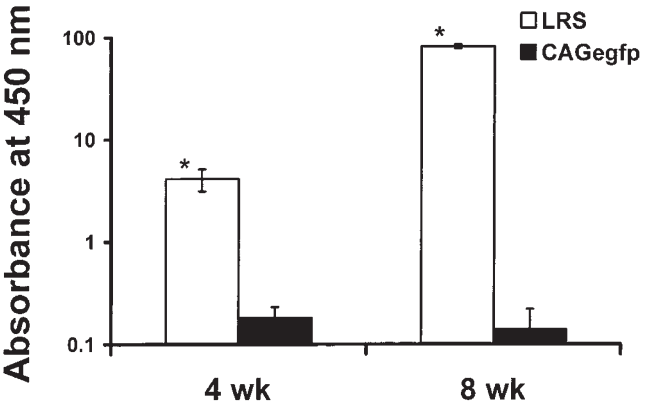

Figure 3 Cell proliferation assay on rAAV-transduced hNPCs. Dissociated hNPCs were sham-transduced or exposed to CAGegfp at an MOI of 10^3. At 4 or 8 weeks after transduction, cells were subjected to a WST-1 proliferation assay. The formazan dye produced by metabolically active cells was quantified by measuring its absorbance at 450 nm, which represented indirectly the number of proliferating cells. Each absorbance value was an average ± s.e. over four repeats. The absorbance at 450 nm is plotted logarithmically on the Y-axis. Open bar, sham-transduced cells; solid bar, CAGegfp-treated. *Values are significantly different from the CAGegfp-treated cells ($P < 0.001$, $n = 4$) analyzed by one-way ANOVA.

rospheres, we designed the following experiment using dissociated cells. Trypsin-dissociated hNPCs were treated with purified CAGegfp at an MOI of 10^3 or an equal volume of lactated Ringer's solution without virus as a control. Cell growth and EGFP expression were monitored daily under an inverted epifluorescent microscope. As shown previously, EGFP green cells were detectable about 1 day after transduction, with an increase in green intensity over the first week, at which point 100% of cells were green labeled. Dissociated single cells from all treatment groups gradually formed spheres, which appeared to grow at a much slower rate when transduced with CAGegfp.

To quantitatively determine the effect of rAAV transduction on hNPCs proliferation *in vitro*, a proliferation assay was conducted at 4 or 8 weeks after transduction on dissociated hNPCs. As shown in Figure 3, sham-treated hNPCs grew about 23-fold faster than CAGegfp-transduced at 4 weeks after treatment. Furthermore, a lack of increase in mitochondrial dehydrogenase activity during a continuous culture period between 4 weeks and 8 weeks indicated that CAGegfp-treated cells ceased proliferation over time. At the latter time point, the number of sham-treated cells reached about 586-fold of that of CAGegfp-transduced.

hNPCs transduced with CAGegfp retain the ability to differentiate into neurons and glia

We next performed differentiation experiments to address whether rAAV transduction affected the ability of hNPCs to differentiate into neurons and glia. Untreated hNPCs differentiated mainly into neurons and astrocytes with little evidence of oligodendrocyte development as shown previously.[19] Neurons and astrocytes were confirmed by their morphology, as well as the IF labeling with antibodies specifically against neuronal markers (TUJ1 and NeuN) and the astrocyte marker (GFAP), respectively (Figure 4a–c). Furthermore, numbers of total cells, green EGFP-labeled cells, red TUJ1 neurons or red GFAP astrocytes were counted from two separate sets of experiments with three coverslips in each set. In the first set, neurospheres were directly dropped on to poly-D-lysine and laminin-coated coverslips with DMEM containing B27 supplement. Under this differentiation condition, 5.1 ± 1.0% and 86.9 ± 12.9% of cells were labeled with TUJ1 and GFAP marker, respectively in untransduced hNPCs. In contrast, 39.8 ± 4.6% and 49.6 ± 10.2% of hNPCs became TUJ1- and GFAP-positive cells, respectively, in the second set of experiment in which thoroughly rinsed neurospheres were then plated for differentiation. Apparently, the trace amount of growth factors, such as EGF, in the first set of experiments was responsible for the differentiation mainly toward an astrocyte lineage. This differentiation profile was unaltered in hNPCs pre-transduced with CAGegfp for 3 weeks (Figure 4d–f) or 3 months (data not shown). For example, cells treated with AAV vectors for 3 weeks resulted in 6.7 ± 2.3% of neurons and 90.3 ± 11.5% astrocytes in the first set of experiments, while 35.8 ± 5.9% of neurons and 55.7 ± 15.9% of astrocytes resulted in the second set. Statistical ANOVA showed no significant differences between the control and AAV-transduced groups.

rAAV transduction in differentiated hNPCs

It is well known that rAAV vectors can transduce both dividing and nondividing cells. In order to confirm their transduction in postmitotic hNPCs, cells were differentiated first by withdrawing growth factors and cultured in B27-supplemented medium. Seven days later, differentiated hNPCs were exposed to CAGegfp for another 7 days. As shown in Figure 5, these cells were still highly susceptible to rAAV transduction. For those areas with cells migrated out from the sphere cores, nearly 100% cells turned green. In addition, IF labeling with a neuron-specific TUJ1 antibody revealed the neuronal phenotype in some of the EGFP-expressing cells (Figure 5b). About 32.2 ± 11.1% of the EGFP-positive cells were also double-labeled with the TUJ1 neuronal marker, which was in the

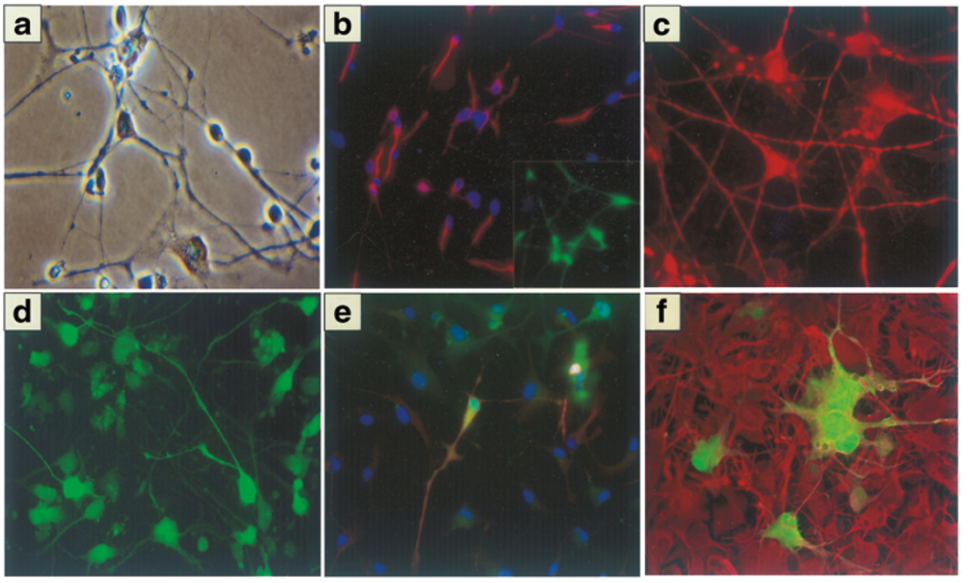

Figure 4 *Differentiation of sham-treated (a–c) or CAGegfp-transduced (d–f) hNPCs in vitro. hNPC spheres were cultured in DMEM/F12/20 ng/ml EGF/20 ng/m bFGF/5 μg/ml Heparin/N2 for 23 weeks. At the time of growth factor withdrawal, cells were plated on glass coverslips precoated with 0.01% poly-D-lysine and 1 μg/ml laminin, and then subjected to differentiation in DMEM/F12/B27 for 14 days. (d–f) Cells were exposed to CAGegfp at an MOI of 10^3 for 3 weeks, and then differentiated for 14 days. (b, c and e, f) Cells were subjected to an indirect IF labeling. (b and e) TUJ1 monoclonal antibody at a 1:500 dilution; insert, NeuN monoclonal antibody at 1:100. (c and f) GFAP polyclonal antibody at 1:1000. The Alexa 594-conjugated secondary antibody (red in b, c, e and f), as well as the Alexa 488-conjugated secondary antibody (green in b, insert) were diluted at 1:200. Cell nuclei were counterstained in blue with 1 μg/ml DAPI (b and e). (e and f) Yellow cells resulted from a combination of EGFP natural green fluorescence and an IF labeling with red Alexa 594.*

range of neuronal differentiation from untreated hNPCs (see above).

Transplantation of CAGegfp-modified hNPC in adult rat spinal cord

Based on previous studies indicating that adult CNS environment, at least in non-neurogenic regions, might not be able to provide sufficient cues to direct neuronal differentiation from stem cells,[41,42] we conducted differentiation *in vitro* before hNPC transplantation into the adult rat spinal cord. In addition, two slightly different approaches were applied including: (1) hNPCs were subjected to CAGegfp transduction for 7 days first and then to an 8-day differentiation; and (2) hNPCs were differentiated first for 4 days, which was followed by CAGegfp exposure for another 4 days. Each group contained four rats for a quantitative analysis. Just before grafting, cells were gently trypsinized and dissociated mechanically into single cell suspension, and stored on ice till use. Over 90% of cells were alive according to a trypan blue exclusion assay. To inhibit immune rejection, male Sprague–Dawley rats were treated with immunosuppressor, 100 μg/ml cyclosporine, in drinking water for 3 days before surgery and throughout the experimental period till their termination. About 10^4 cells in 5 μl of DMEM were transplanted into the grey matter of spinal cord at L1. The leftover hNPCs were plated on to culture dishes and then examined under an epifluorescent microscope. Green cells in 10 randomly chosen fields (more than 200 cells) were counted and compared over the total cell numbers in each field. With the MOI of 4×10^3 we used, about 80% transduction efficiency was obtained. After surgery, animals were kept on cyclosporine treatment for 2 weeks till death. Animals were healthy except some limping during the first 3–5 days after surgery.

Spinal cords were collected and cryosectioned longitudinally at 20–30 μm. Grafts were examined directly under a Nikon epifluorescent microscope. With the assistance of the EGFP marker, we could easily locate grafted cells with green labels. Only 2 weeks after transplantation, neuron-like cells with long processes and some degree of spreading were observed in hNPCs grafts with a short period of pre-differentiation *in vitro* (Figure 6). In addition, the exogenous EGFP marker allowed us to trace processes from the graft readily as shown in Figure 6b and e. Some of the processes were identified 0.4 mm away from graft and longer than 0.55 mm in length (Figure 6b). Our data indicated that these differentiated hNPCs were able to be incorporated into the host environment in rat spinal cord.

To confirm that hNPCs differentiated or matured further into neurons in adult rat spinal cord, sections were also subjected to an indirect IF labeling using NeuN or TUJ1 neuron-specific antibodies, GFAP antibody for astrocytes, or GalC for oligodendrocytes. Many EGFP green cells were positive for NeuN (Figure 6c) and TUJ1 (Figure 6f) IF labeling examined either by an epifluorescent microscope or a confocal microscope (Figure 7a–c). Under the conditions we used, fewer EGFP green cells were double-labeled with a GFAP antibody (Figure 7d–e). Their neuronal and astrocyte phenotypes were very similar to differentiated hNPCs *in vitro*. No oligodendrocytes were observed with green labels. On the other hand, some of the green cells were unlabeled with any of the above markers, which suggested their undifferentiated status 2 weeks after transplantation. In a trial experiment using hNPCs treated with another type of AAV vector containing a β-galactosidase reporter gene, no fluorescent cells were observed after transplantation into rat spinal cord (data not shown).

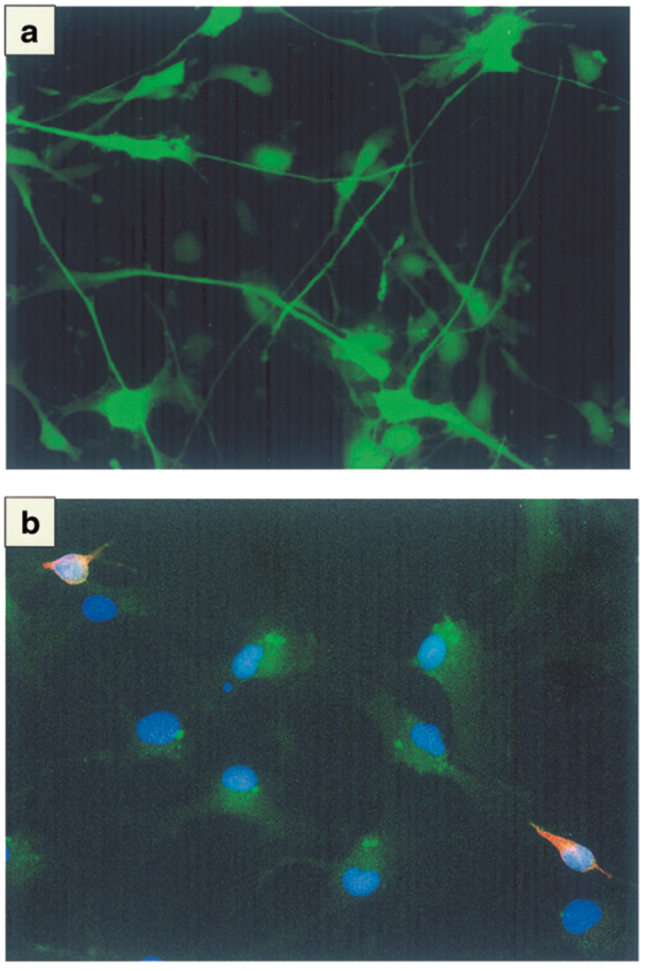

Figure 5 *EGFP transgene expression in differentiated hNPCs in vitro. 25-week cultured hNPCs underwent a 7-day differentiation, and were then exposed to CAGegfp (MOI of 2 × 10³) for 7 days. (a) A live image of differentiated cells expressing EGFP. (b) Paraformaldehyde-fixed cells subjected to an indirect IF labeling with a neuron-specific TUJ1 monoclonal antibody (diluted 1:500) and Alexa 594-conjugated secondary antibody at 1:200. Cell nuclei were counterstained in blue with 1 μg/ml DAPI.*

To further determine the percentage of neurons or astrocytes derived from hNPCs in rat spinal cord, three sections separated at least by 150 μm from each rat were IF-stained for either neuronal or astrocyte marker. The percentages of each cell type were then averaged over four rats in each group. From animals using the hNPCs that were treated with CAGegfp and then differentiated, 42.9 ± 11.7% and 7.2 ± 3.3% of AAV-transduced green hNPCs became neurons and astrocytes in rat spinal cord, respectively. Similar results, 48.2 ± 15.4% of neurons and 6.8 ± 2.9% of astrocytes, were obtained from animals grafted with hNPCs that were differentiated and then transduced with CAGegfp vectors. In a parallel *in vitro* study, a portion of the treated cells used for transplantation were also plated on to a coated glass coverslip and subjected to further differentiation in B27. Ten days later (due to cell viability), cells were fixed for IF staining. The percentage of neurons among total EGFP cells was about 10.8 ± 3.5%. This low yield of neurons *in vitro* compared with the high yield of neurons *in vivo* from the same batch of AAV-treated hNPCs suggested that the host spinal cord might contain additional neurotrophic factors to support further neuronal differentiation and maturation as shown in our previous observations.[43]

Discussion

In this study, we demonstrated for the first time that rAAV vectors could deliver foreign genes into cultured human NPCs efficiently and stably. More importantly, these AAV-transduced hNPCs retained their capability to differentiate into neurons and glia both *in vitro* and after transplantation into adult rat spinal cord *in vivo*.

Using the rAAV vector, CAGegfp, we were able to express EGFP in hNPCs. The EGFP transgene expression mediated by rAAV vectors was gradually taking place in hNPCs with a few faint green cells shown 1–2 days after treatment, which reached a peak in about 2 weeks. This delay of rAAV-mediated transgene expression in hNPCs is consistent with the previous findings of rAAV transduction in other types of mammalian cells,[44–46] and, due to a rate-limiting step, is believed necessary to convert single-stranded rAAV viral genome into expressible double-stranded form.[44,45,47–49] Further time-course and dose–response studies indicated that EGFP transgene expression in hNPCs was dependent on cell stage, time period after exposure, as well as dosage of rAAV vectors. For medium-sized hNPC spheres, the percentage of EGFP-expressing cells increased about three- to five-fold at various time-points when the amount of rAAV vectors was increased by 100-fold. Apparently, the increase in EGFP-positive cells was not directly proportional to the increment of rAAV particles applied. A likely explanation is that cells in the center of the hNPC spheres might not be exposed readily to rAAV transduction. This hypothesis was supported further by the fact that a 100% efficiency was observed in dissociated hNPCs with fewer rAAV vectors (MOI of 10^3), while only a 86% efficiency was achieved in spheres with rAAV at a higher MOI of 5×10^3. In addition to the above parameters, the efficiency of EGFP expression in hNPCs was also highly dependent on promoters. Besides the CAG promoter reported in this study, other promoters that we tested *in vitro* included a human viral CMV promoter,[37] a rat neuron-specific enolase (NSE) promoter,[50] a human elongation factor 1α-subunit (hEF-1α) promoter,[51,52] and a rat calmodulin gene II (CaM II) promoter.[53] All these promoters were able to express EGFP in both dividing and differentiated hNPCs (unpublished observation). Using the same dosage (MOI of 10^3), the potencies of these promoters to drive EGFP expression in hNPCs were ranked as CAG ⩾ CMV >> hEF-1α > NSE = CaM II (unpublished observation). Based on this initial experiment, we decided to use the CAG promoter for the remaining *in vitro* and *in vivo* studies.

rAAV vectors were known to deliver foreign gene stably into many types of mammalian cells including hematopoietic cells, muscle cells and neurons.[31,54,55] The long-term expression of transgenes mediated by rAAV vectors has been attributed to their ability to integrate transgenes into host chromosome,[38,56–59] as well as the formation of episomally stable and expressible rAAV multimers.[47,60] In this study, we also showed that the EGFP expression mediated by rAAV vectors was stable in hNPCs for at least 4 months, the longest time-point examined thus far. However, the percentages of EGFP-positive cells declined over 2 months of culture by 80%

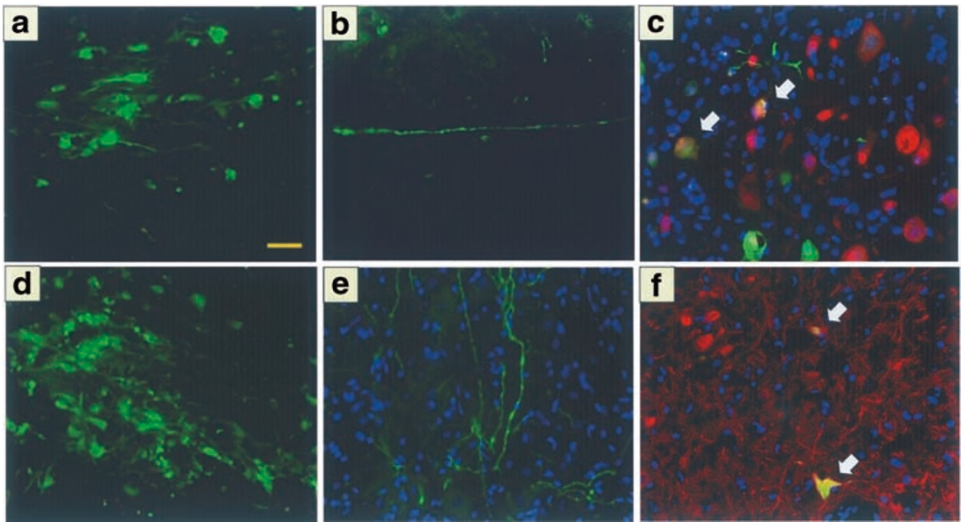

Figure 6 CAGegfp-transduced hNPC grafts in rat spinal cord. (a–c) hNPCs were transduced with CAGegfp at an MOI of 4×10^3 and then subjected to differentiation for 8 days in vitro before transplantation. 10^4 cells/5 μl were injected into left L1 of spinal cord of a male adult rat. (d–f) hNPCs were differentiated for 4 days and then treated with CAGegfp for 4 days before grafting. Two weeks later, spinal cords were cryosectioned longitudinally at 20 μm. EGFP green-labeled hNPCs were immediately observed under a fluorescent microscope. (a and d) Transplanted EGFP-expressing cells near the injection site. (b and e) Elongated fibers from transplanted hNPCs are observed 0.4 mm and 0.1 mm away from the injection site, respectively. (c and f) Triple-labeled hNPCs including immunofluorescent (red), EGFP (green), and DAPI counterstaining (blue). (c) Immunofluorescent labeled with a neuron-specific monoclonal antibody, NeuN, at 1:200. (f) Labeled with another neuron specific marker, TUJ1 monoclonal antibody at 1:500. Arrow, yellow cells indicate the EGFP expressing neurons from the hNPC origin. Scale, 40 microns.

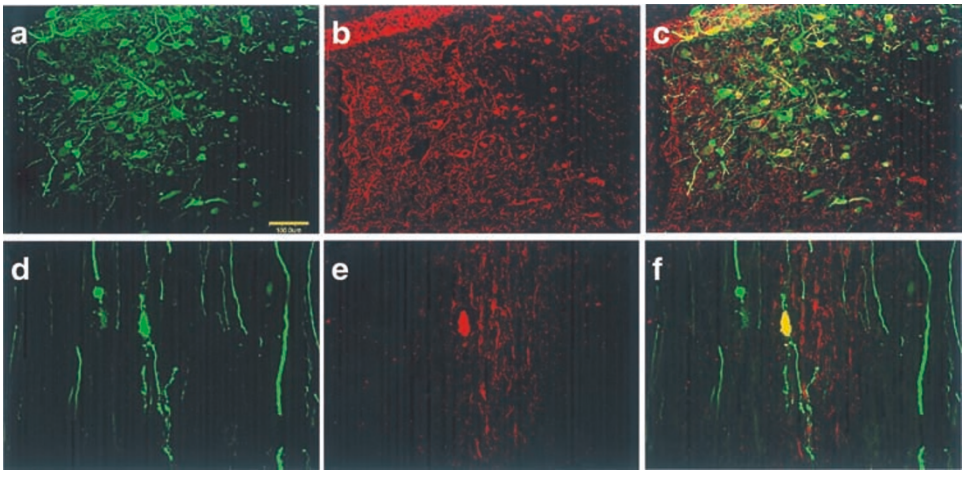

Figure 7 Confocal images of neurons and astrocytes differentiated from CAGegfp-transduced hNPC in rat spinal cord. (a and d) Green cells and fibers are grafted hNPCs expressing the EGFP marker. (b and e) The same sections as (a) and (d) immunofluorescent labeled with a neuron-specific TUJ1 antibody and an astrocyte-specific GFAP antibody, respectively. (c and f) Overlaid images showing double-labeled neurons and astrocytes (yellow), respectively. Scale, 100 microns.

at a low MOI and by 50% at a high MOI, which are consistent with the previous report of rAAV transduction in human hematopoietic cells.[61] Since AAV-mediated integration in mammalian cells is only around 1–5%,[62] the decrease of EGFP-expressing cells over time might be due to the continuous loss of episomal vector genomes during cell division. Therefore, proper selections may be required should cloning of hNPCs be necessary to express a particular gene.

Alternatively, the decrease in the EGFP-expressing cells in a growing sphere might be a combination of two processes: (1) a continuous proliferation of untransduced cells; and (2) a slowing and eventually cessation of growth of cells expressing the EGFP transgene. This is based on our observation of a slower growth in CAGegfp-transduced cells when compared with the sham-treated, especially those with the rAAV at a higher MOI. In addition, we performed a WST-1 proliferation assay on dissociated hNPCs with 100% of cells becoming EGFP-positive after rAAV transduction. Our results indicated that cells expressing EGFP either ceased to proliferate or grew very slowly under regular culture conditions. We have also observed some spontaneous differentiation in CAGegfp-transduced hNPC spheres, despite the continuous supply of growth factors. Other than these, no apparent cytotoxicity was detected since the transduced hNPCs maintained their viability for at least 3 months. In addition, long-term (eg 3 months in this experiment) cul-

tured EGFP-expressing hNPCs retained their capability to differentiate into both neurons and glial cells. Further quantitative studies demonstrated that CAGegfp transduction did not alter the differentiation profile of neurons and astrocytes as shown in untransduced hNPCs. The reason(s) for CAGegfp-induced cessation in hNPC growth *in vitro* is unknown. One of the possible candidates is the EGFP transgene product, which may affect mitosis and/or differentiation of hNPCs at least under the culture conditions that we have used. A similar observation was reported earlier by Martinez-Serrano *et al*,[63] who used a retroviral vector containing EGFP to transduce human NSCs, although a possibility of capsid proteins on the rAAV viral particles or the trace amount of cellular proteins in viral preparation to affect proliferation of hNPCs could not be completely excluded.

NSCs are very plastic and possess multipotential to become neurons, astrocytes and oligodendrocytes. This differentiation plasticity of NSCs is both intrinsically determined and radically influenced by the extracellular environment.[16] Although establishing a clone of hNPCs genetically modified by a specific gene may be desirable for many applications, it may not be possible to maintain either their normal proliferation as in the case of EGFP, or their differentiation potential. Accumulated evidence indicated that an accurate sequential exposure of neural stem cells to inductive factors or cues might be critical for them toward a certain cell phenotype. Delivering foreign genes such as neurotrophic factors in stem cells at the dividing stage may have an impact on their cell fate.[16,64] Therefore, it may be necessary for some applications that hNPCs should be differentiated first before genetic modification. To this end, we conducted both *in vitro* and *in vivo* experiments to determine the transduction of rAAV in differentiated hNPCs. Our data showed that both neurons and glial cells differentiated from hNPCs expressed the foreign EGFP gene after exposure to CAGegfp vectors *in vitro*. Furthermore, these hNPC-derived neurons and glia cells survived and continued to express EGFP after transplantation in rat spinal cord. rAAV vector-mediated foreign gene delivery into differentiated neurons may be very useful in those cases that require a specific lineage of neural cells before transplantation.

In an initial trial experiment, we observed limited differentiation in hNPC grafts 2 weeks after transplanting undifferentiated cells into the grey matter of the spinal cord in adult rats, a phenomenon also reported by others.[65] Therefore, a procedure of differentiation *in vitro* before transplantation was carried out for all the *in vivo* experiments reported here. hNPCs were subjected to either rAAV transduction first followed by differentiation, or differentiation followed by transduction. Both approaches yielded significantly more differentiated neurons at 2 weeks after transplantation in rat spinal cord. In addition, prolonged fibers from transplanted hNPC neurons were observed. Further experiments are ongoing to determine whether these rAAV-transduced hNPCs can express EGFP transgene for a longer period of time after transplantation.

In summary, our study demonstrated for the first time that rAAV vectors can efficiently transduce both proliferating and differentiated hNPCs. Most importantly, transduced hNPCs were still able to undergo multilineage differentiation toward neurons and glial cells without apparent cytotoxicity both *in vitro* and after transplantation in rat spinal cord *in vivo*. Human NSCs genetically modified with the safe and efficient rAAV vectors will provide a powerful tool for studies of neurological development, as well as for treatment of neurological disorders including neurodegenerative diseases and neurotrauma.

Materials and methods

Culture of hNPCs

Human embryonic cortex (8 week after conception) was collected in the UK in strict accordance with both the Department of Health guidelines and the recommendations of the Polkenhorn Committee. Following trypsinization and seeding into flasks at 400 000 cells per ml, cells formed aggregates which developed into neurospheres in response to a combination of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and heparin as described previously.[19] Neurospheres were passaged every 2 weeks using a novel chopping method that maintains cell/cell contact and allows the continual growth of the cells. Following 15 weeks of growth in culture, a batch of spheres was stored in ice-cold embryonic hibernation medium (Life Technologies, Gaithersburg, MD, USA), and then shipped to the University of Texas Medical Branch (UTMB). Upon arrival, cells were transferred into a T75 flask containing DMEM:F12 (3:1) supplemented with N2 medium (Life Technologies), 20 ng/ml EGF (R&D Systems, Minneapolis, MN, USA), 20 ng/ml bFGF (R&D Systems) and 5 μg/ml heparin (Sigma, St Louis, MO, USA). Maintenance of hNPCs *in vitro* was carried out based as previously described.[19] Cells were incubated with 5% CO_2 at 37°C. Approximately half of the growth medium was changed every 3–4 days. Once every 2 weeks, large spheres were chopped into smaller pieces using two No. 10 surgical blades, and cells were split 1:2. About 7 days after chopping, hNPCs were cryopreserved in DMEM with 20% fetal bovine serum (FBS; Life Technologies) and 10% DMSO (Sigma), cooled slowly in a cryocontainer with isopropanol, stored first in a −80°C freezer and then in liquid nitrogen.

For differentiation, hNPC spheres were seeded on glass coverslips (Carolina Biological Supply Company, Burlington, NC, USA) precoated with 0.01% poly-L-ornithine (Sigma) and 0.2–1 μg/cm^2 laminin (Sigma). hNPCs were incubated in DMEM/F12 (3:1) supplemented with B27 (Life Technologies) for 8 to 14 days, with a change of half volume of medium every 3–4 days.

Construction of recombinant AAV (rAAV) plasmid

The enhanced green fluorescent protein (EGFP) was used as a marker to determine the efficiency of AAV transduction in hNPCs. pTR-UF2, kindly provided by N Muzyczka (University of Florida), is an rAAV plasmid containing AAV2 ITRs.[66] pTRΔS is a derivative of pTR-UF2 by *Sal*I restriction digestion to delete the neo gene cassette. The 0.7-kb EGFP gene was released from pEGFP (Clontech Laboratories, Palo Alto, CA, USA) by *Kpn*I and *Not*I digestion, and then inserted into pTRΔS, which was termed as pTRegfp. Finally, a 1.2-kb CAG promoter[39] excised from pBacMam-3 (Novagen, Madison, WI, USA) by *Bsm*BI and *Kpn*I was ligated into pTRegfp. The resulting rAAV was designated as pCAGegfp.

Preparation of rAAV viral stocks

The packaging of rAAV vectors was performed based on others and our own protocols,[37,38,67,68] with some modifications. A three-plasmid co-transfection method developed by Xiao *et al*[67] was applied to raise viral stock efficiently, yet exclude the potential contamination of toxic adenovirus. In this method, all the AAV *trans*-acting factors were provided by an AAV helper plasmid – pXX2, and an AV helper plasmid – pXX6. Both plasmids were kindly provided by X Xiao (University of Pittsburgh). The crude viral preparation was then purified through heparin affinity chromatography.

293 cells were cultured in 150 mm dishes with DMEM/10% FBS at 37°C, 5% CO_2. As soon as they reached 80% confluence, cells were co-transfected with pCAGegfp, pXX2 and pXX6 using a standard calcium phosphate precipitation method. After one brief rinse with DMEM, OptiMEM (Life Technologies)/10% FBS/120 μM chloroquine was added to the cells. This was followed by adding 2.5 ml per plate of a DNA–calcium phosphate cocktail, which contained three plasmids at the molar ratio of 7:2:4, 125 mM $CaCl_2$ and 1 × HBS (2.5 M NaCl, 0.25 M HEPES, 75 mM Na_2HPO_4, pH 7.1). Cells were cultured with 5% CO_2 at 37°C for 17 h, and changed with OptiMEM/10% FBS. Two days after transfection, cells and medium were collected, centrifuged at 1140 *g* for 15 min, and then resuspended in 150 mM NaCl/20 mM Tris pH8.0 at 5×10^6 cells/ml. The cell suspension was further treated with 0.54% deoxycholate (Sigma) and 50 U/ml Benxonase (Sigma) at 37°C for 1 h. Following centrifugation at 3000 *g* at room temperature for 20 min, supernatants were subjected to a cycle of freeze–thaw, and then centrifuged again at 10 000 *g* at 4°C for 30 min. The supernatant was collected, filtered through a 1-μm disk filter (Fisher, Pittsburgh, PA, USA), and then run by gravity through a heparin agarose type I column (Sigma) pre-equilibrated with phosphate buffer saline/1 mM $MgCl_2$/2.5 mM KCl (PBS-MK). After four washes with 5 ml PBS-MK each, rAAV viruses were eluted by 9 ml of 1 M NaCl/PBS-MK. The first 2 ml was discarded. The following 7 ml was collected, desalted by running through a Centricon Plus-20/Biomax-100 (Fisher) with four changes of lactated Ringer's solution, and concentrated by centrifugation at 3000 *g* at room temperature till reaching a desired volume. The 7 ml of elution was collected based on a PCR confirmation of rAAV-positive signals in serial fractions.

rAAV titers were determined by a DNA dot-blot analysis.[69] Briefly, 1 μl or 5 μl of affinity column-purified CAGegfp was subjected to 5 μg/μl proteinase K digestion. The viral DNA was purified by a GeneClean Spin Kit (BIO 101, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA standard was prepared using a two-fold serial dilution of the pCAGegfp plasmid in a range of 0.3–76.8 ng. After mixing with 0.4 ml of 0.4 M NaOH/10 mM EDTA, both viral and standard DNA were heated at 100°C for 5 min, cooled on ice for 2 min, and then loaded on to a nylon membrane through a dot-blot manifold apparatus. The membrane was hybridized with a α-^{32}P-CAG specific probe in 6 × SSPE/Denhardt/0.5% SDS/100 μg/ml sperm DNA at 65°C for 18 h. The radiolabeled probe was made using a Random Primers DNA Labeling System according to the manufacturer's instruction (Life Technologies). Following one wash with 2 × SSC (65°C, 30 min) and twice with 0.1% SDS/2 × SSC (RT, 15 min), the membrane was exposed to an X-ray film for autoradiography. The hybridization signals were quantitatively measured by a computerized ChemiImager 4400 Low Light Imaging System (Alpha Innotech, San Leandro, CA, USA). Titers of the CAGegfp vectors used in this study were around $10^{11–13}$ viral particles/ml.

hNPCs under proliferation or differentiation as described above were exposed to CAGegfp rAAV vectors at various dosages. Media were changed every 3 days. Expression of EGFP transgene was monitored directly under a Nikon inverted epifluorescent microscope.

WST proliferation assay for hNPCs

0.025% Trypsin-dissociated hNPCs were plated at 3.6×10^4 cells/cm^2 in a 96-well plate, and subjected to rAAV vector (at MOI of 10^4) or sham (lactated Ringer's solution) transduction. At the indicated intervals after treatment, proliferation of hNPCs (n = 4) was determined using WST-1 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. The activity of mitochondrial dehydrogenase to convert tetrazolium salts into formazan, which represents the number of live cells, was measured in this simple and sensitive assay. Briefly, 100 μl of each cell culture in a 96-well plate was incubated with 10 μl WST-1 for 4 h at 37°C with 5% CO_2. After shaking for 1 min, the absorbance at 450 nm was measured using a μQuant microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Culture medium without cells were included as controls for each well.

hNPC transplantation in rat spinal cord

hNPCs used for transplantation were either differentiated for 8 days *in vitro* as described above, or left without differentiation. CAEegfp transduction (MOI of 4×10^4) was performed either 7 days before differentiation or 4 days after differentiation. Immediately before transplantation, cells were incubated in 1:2 diluted 0.05% trypsin/0.53 mM EDTA (Sigma) in CMF-PBS for 5–10 min at RT. After adding DMEM medium containing 1 mg/ml trypsin inhibitor (Sigma), 0.6% glucose and 0.04% DNase I (Sigma), cells were mechanically dissociated by passing through a 18-gauge needle first and then a 25-gauge needle. The number of living cells was determined by a trypan blue exclusion assay. Cells with higher than 90% viability were centrifuged at 200 *g* for 10 min and resuspended in DMEM/0.01% DNase I in a density of 10^4 cells/5 μl, and stored on ice till grafting. Cell suspensions were pipetted once every hour to maintain dissociation.

The spinal cord transplantation protocol was established according to the NIH guidelines for the care and use of laboratory animals, and approved by the University of Texas Medical Branch IACUC. Male 200–230 g Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were treated for 3 days before surgery with an immunosuppressor NEORAL cyclosporine (Novartis Pharma, East Hanover, NJ, USA) at 100 μg/ml in drinking water, and kept thereafter till the termination of animals. On the day of transplantation, animals were administered intraperitoneally (i.p.) with 10 mg/kg xylazine and 90 mg/kg ketamine for anesthesia. An antiseptic laminectomy was performed at L1 using the last rib as a reference. Under the assistance of an operation microscope, dura was

incised after applying a small amount of 1% lidocaine. The animal was then placed on to a small animal spinal apparatus (David Kopf Instruments, Tujunga, CA, USA), with its dorsal spinous processes fixed by spine clamps. About 10^4 cells/5 μl hNPCs were injected into the left side of spinal cord 1 mm from the midline and 1.5 mm in depth using a 5-μl Hamilton syringe with a 26-gauge needle. The injection speed was controlled at 0.25 μl/min using a microinjection unit (David Kopf Instruments). The needle was then held in place for 1 min before gradually withdrawing. Fascia and muscles were sutured and the skin incision was closed with surgical staples. Animals were administered with 65 000 units sodium penicillin (i.m.) and 4 ml lactated Ringer's solution (i.p.), and kept on a heating pad till awake.

Immunofluorescent (IF) analyses

For fluorescent and IF studies, rats were treated with an overdose of sodium pentobarbital (i.p.) before intracardial perfusion at 65 ml/min with 0.1 M cold PBS and then 400 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.4. Spinal cord fragments (1–2 cm in length) were dissected, postfixed in 4% paraformaldehyde at 4°C overnight, and then immersed in 30% sucrose till sunk (usually 2 days). Tissue blocks were embedded in OCT compound and frozen in liquid nitrogen-cooled isopentane. Serial longitudinal sections at a thickness of 20–30 μm were cut in a cryostat, collected on to glass slides pretreated with gelatin-chromium sulfate, and stored at −70°C. Just before IF labeling, cultured hNPC cells were fixed with 4% paraformaldehyde/PBS for 30 min, and rinsed for three times in PBS, 5 min each.

Cells or sections were incubated for 30 min in 5% bovine serum albumin (BSA) with (for cytoplasmic antigen) or without (for surface antigen) 0.1–0.5% Triton X-100. This was followed by an overnight incubation with primary antibodies at optimal concentrations at 4°C. After three rinses with PBS for 15 min each, samples were incubated with Alexa fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) at optimal concentrations for 1 h at room temperature in the dark. After three × 5-min rinses with PBS, cells or sections were stored under Fluoromount G (Fisher). Some cells or tissue sections were subjected to counterstaining with 1 μg/ml DAPI (Sigma) at room temperature for 5 min, followed by a brief rinse in PBS and then mounted. All the antibodies were empirically tested for their optimal concentrations, as well as centrifuged at 13 500 *g* at 4°C for 2 min before use.

Rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody was used for labeling astrocytes at 1:500 in cells and 1:1000 in sections. For neurons, anti-neuronal nuclei (NeuN) monoclonal antibody and anti-β-tubulin (TUJ1) monoclonal antibody were used at 1:200 and 1:500, respectively. Anti-galactocerebroside (GalC) monoclonal antibody was applied for oligodendrocytes at 1:50. All the primary antibodies were purchased from Chemicon International (Temecula, CA, USA) except for TUJ1 antibody, which was from COVANCE/BabCO (Richmond, CA, USA). Alexa Fluo 594 or Alexa Fluo 488 conjugated goat anti-mouse or goat anti-rabbit IgG (Molecular Probe) were used at 1:200. Fluorescent labeling was examined with DAPI, FITC or Texas Red filters on a Nikon Microphot-FX microscope. Images were captured with a Photometrics Coolsnap FX Monochrome digital camera, and pseudo-colored using a MetaMorph image system (Advanced Scientific, Dallas, TX, USA). Some spinal cord sections were examined using an Olympus Fluoview confocal microscope (Leeds Instruments, Irving, TX, USA).

Quantitative analysis

For *in vitro* study, the number of cells labeled with a specific antibody and the total cells counterstained with DAPI were counted in 10 randomly chosen fields (containing >200 cells) per sample. The percentage of labeled cells was averaged over 10 fields and then further averaged over triplicates. For transplantation study, three sections per animal separated at least by 150 μm from each other were each subjected to immunostaining with a specific antibody. The number of green EGFP-labeled cells and the number of double-labeled cells were counted in each section. The percentages of double-labeled cells were averaged from four animals in each experimental group.

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