Glial cell line-derived neurotrophic factor-secreting human neural progenitors show long-term survival, maturation into astrocytes, and no tumor formation following transplantation into the spinal cord of immunocompromised rats

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Human neural progenitor cells (hNPCs) derived from the fetal cortex can be expanded *in vitro* and genetically modified through lentiviral transduction to secrete growth factors shown to have a neurotrophic effect in animal models of neurological disease. hNPCs survive and mature following transplantation into the central nervous system of large and small animals including the rat model of amyotrophic lateral sclerosis. Here we report that hNPCs engineered to express glial cell line-derived neurotrophic factor (GDNF) survive long-term (7.5 months) following transplantation into the spinal cord of athymic nude rats and continue to secrete GDNF. Cell proliferation declined while the number of astrocytes increased, suggesting final maturation of the cells over time *in vivo*. Together these data show that GDNF-producing hNPCs may be useful as a source of cells for long-term delivery of both astrocytes and GDNF to the damaged central nervous system. *NeuroReport* 25:367–372 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a disease in which motor neurons die due to an unknown mechanism. Two out of every 100 000 people are afflicted with ALS, summing to 35 000 people in the USA and UK alone [1]. Patients suffer muscle twitching, cramping, stiffness, slurred speech, eventual paralysis, and ultimately death due to respiratory failure [2]. The diseased environment within the brain and spinal cord, including endogenous astrocytes, may play a leading role in the death of the motor neurons [3–5].

Replacing the motor neurons lost in ALS to reconnect the neural circuit from the brain to the muscle is very complicated. A more practical alternative may be to protect the remaining motor neurons by introducing supportive cells and growth factors. Human neural progenitor cells (hNPCs) derived from the fetal cortex can be expanded *in vitro* for up to 50 passages with a stable karyotype and survive following transplantation into the brain or spinal cord of rodents, pigs, and primates [6–8]. Furthermore, hNPCs can be genetically modified to produce growth factors that have neuroprotective potential [9]. We have previously shown that transplanting hNPCs over passage 20 yields astrocytes *in vivo*, providing nondiseased cells that are allogenic and neuroprotective [10–12]. In addition, we have shown that hNPCs can be modified to stably secrete glial cell line-derived neurotrophic factor (GDNF) *in vivo* [13]. This two-tiered approach using hNPCs to provide astrocytes and the neuroprotective growth factor GDNF proved sufficient to rescue the microenvironment and preserve dying motor neurons in an animal models of ALS [14,15].

We generated a master cell bank (MCB) of hNPCs under current good manufacturing practice (cGMP) that we then transduced with cGMP-grade lentivirus to create research and clinical-grade lots of hNPCs stably secreting GDNF ($hNPCs^{GDNF}$). We are currently completing the first of five steps required to bring these cells to the clinic under a phase 1/2a trial. The first step is to transplant the research lot of $hNPCs^{GDNF}$ into wild-type and $SOD1^{G93A}$ rats, a transgenic model of ALS, to determine the optimal effective dose and maximum feasible dose. Next, the clinical lot of $hNPCs^{GDNF}$ will be used for safety, toxicity, and tumorigenicity testing in immune-compromised nude rats and for a surgical technique safety trial in

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Yucatan mini-pigs. The final goal is Federal Drug Administration approval to undergo an 18 patient trial of unilateral lumbar transplants with systematic clinical assessment over a 12-month period.

An obvious concern when transplanting neural stem or progenitor cells is the risk of cell overgrowth and/or tumor formation. While we have not seen tumor formation following human progenitor cell transplantation into the $SOD1^{G93A}$ transgenic rat, this ALS model does not allow for long-term graft characterization due to the short lifespan of diseased animals. Here, we transplanted our research grade $hNPCs^{GDNF}$ into the spinal cord of immunocompromised athymic nude rats and performed analysis of cell survival, proliferation, phenotype, and GDNF production at 1 month and 7.5 months post-transplantation.

Materials and methods

Derivation, expansion, and banking of hNPCs

Eight-week-old postmortem fetal tissue was collected by Dr Guido Nikkhah (Germany) with institutional review board approval by his institution and by the University of Wisconsin-Madison, where the resulting cell line was generated. The intact primary cortical mantel was identified, isolated, and dissociated into a single cell suspension. The resulting hNPC line was expanded as free floating spheres, termed neurospheres, in expansion medium (Stemline Neural Stem Cell Expansion Medium, S3194; Sigma-Aldrich, St. Louis, Missouri, USA), 100 ng/ml human recombinant epidermal growth factor (GF003-AF; EMD Millipore, Billerica, Massachusetts, USA), 10 ng/ml leukemia inhibitory factor (LIF1010; EMD Millipore). At passage 19, a bank of cGMP-grade hNPCs, termed the MCB, was generated and cryopreserved by the University of Wisconsin-Madison Waisman Center Bio-Manufacturing Facility. The MCB can be sourced to generate cGMP cell lots for experimentation and clinical transplantation. The hNPCs were dissociated using a combined enzymatic (TrypLE Select, 12563-011; Life Technologies, Carlsbad, California, USA) and glass pipette mechanical dissociation strategy followed by freezing at a single cell concentration of 5.0e6 cells/ml in freezing media (Cell Freezing Medium-DMSO Serum Free, 1x, C6295; Sigma-Aldrich). Six vials of the MCB were then thawed, expanded, transduced with a cGMP-grade lenti virus-SIN-WPRE-mPGK-GDNF (Indiana University Vector Production Facility) at passage 26, further expanded and banked at passage 29 as a 382 vial research lot (Cedars-Sinai Medical Center IRB 21505, IRB-SCRO 29996, 22279). The lentiviral construct encoding GDNF is characterized by the mouse phosphoglycerate kinase 1 promoter, which provides constitutive expression of GDNF, as well as post-translational cis-acting regulatory element of the woodchuck hepatitis virus (WPRE) as it has been shown to significantly enhance transgene expression.

Generation and characterization of $hNPC^{GDNF}$

From the hNPC MCB, cells at passage 26 were transduced with a cGMP-grade lenti virus-SIN-WPRE-mPGK-GDNF (Indiana University Vector Production Facility) following previously described methods [9]. Briefly, neurospheres were dissociated to single cells, incubated for 12 h in lentivirus at 125 ng p24 capsid protein/million cells and then fresh expansion media was added to dilute the virus. Neurospheres reaggregated within 24–48 h and stable GDNF gene expression was verified by immunocytochemistry at 1 and 5 weeks postinfection. For *in vitro* differentiation, cells were dissociated, plated onto laminin-coated glass coverslips for 7 days, fixed with 4% paraformaldehyde (PFA), and then stained with antibodies against glial fibrillary acidic protein (GFAP) (Z0334; Dako, Carpinteria, California, USA; 1/500), GDNF (BAF212; R&D Systems, Minneapolis, Minnesota, USA; 1/250), and a DAPI nuclear counterstain (D1306; Life Technologies).

Cell preparation for transplantation

Research lot vials were thawed, rinsed with 2.6% Pulmozyme (Genentech, San Francisco, California, USA)/transplantation medium (buffer solution containing glucose), counted, centrifuged, resuspended at the appropriate transplantation concentration in transplantation media and stored on ice until completion of surgery. Cell viability before and after surgery was confirmed using trypan blue exclusion counts and by plating the cells on laminin-coated coverslips for 24 h before fixation.

Spinal transplantation of cells

Male athymic nude rats (Hsd:RH-Foxn1rnu; Harlan Laboratories, Indianapolis, Indiana, USA) at 8 weeks (240–280 g) of age were transplanted with 2 µl of research grade $hNPC^{GDNF}$ in five distinct sites, 1 mm apart at a concentration of 60 000 cells/µl. Briefly, rats were anesthetized with isofluorane, administered analgesic drugs (buprenorphine and carprofen), and transferred to a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) where the 12th rib of the rat was identified and an incision was performed in the skin and muscle to expose the lumbar vertebrae. A hemilaminectomy was performed on the side of the surgery to expose the spinal cord followed by a dura incision. Cells were loaded into a 45° beveled glass micropipette connected to a 10 µl Hamilton syringe and a microinjection pump for injection directly into the parenchyma (0.8 mm mediolateral, 1.8 mm dorsoventral) at a rate of 1 µl/min. The use and maintenance of rats were performed in accordance with the Guide of Care and Use of Experimental Animals of the American Council on Animal Care and the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center (IACUC 4260).

Tissue collection and histology

Rats were anesthetized and transcardially perfused with 0.9% NaCl and fixed with 4% PFA [1224SK-SP; Electron

Microscopy Sciences (EMS), Hatfield, Pennsylvania, USA]. Tissues were collected, postfixed overnight in 4% PFA, and transferred into 30% sucrose for 48 h before sectioning (35 μm) on a sliding microtome (SM2010R; Leica, Wetzlar, Germany). The side contralateral to surgery was identified by notching the dorsal horn. Every 12th section sample of the lumbar spinal cord was immunostained according to standard techniques with the following Stemcells Inc. (Palo Alto, California, USA) human-specific antibodies against Ku80 (SC101, 1/200), GFAP (SC123, 1/2000), and cytoplasm (SC121, 1/2000). Antibodies against Ki67 (VP-K451; Vector Laboratories, Burlingame, California, USA; 1/100), nestin (ABD69; EMD Millipore; 1/10 000), choline acetyltransferase (ChAT) (AB144P; EMD Millipore; 1/200), and GDNF (BAF212; R&D Systems; 1/250) were also used. Sections were stained with fluorophore-coupled secondary antibodies Alexa-488 or Alexa-594 (multiple variants; Life Technologies; 1/1000) and counterstained with DAPI (D1306; Life Technologies) or with 3,3-diaminobenzidine peroxidase kit with nickel enhancement (SK-4100; Vector Laboratories).

Stereology and immunohistological quantifications

Stereological quantification was performed using the optical fractionator method (MBF Biosciences, Williston, Vermont, USA). For SC101/Ki67 and nestin/GFAP cell counts, the ipsilateral spinal cord sections were individually traced. SC101, Ki67, nestin, and GFAP-positive cells were counted at a $\times 60$ magnification, with parameters of the distance between counting frames (500 μm), the counting frame size (75 μm × 75 μm), the dissector height (23 μm), and the guard zone thickness (2.5 μm).

Statistical analysis

Prism software (GraphPad Software, La Jolla, California, USA) was used for all statistical analyses. All counting data from immunocytochemical/histochemical analyses and cell survival were expressed as mean values ± SEM and analyzed by two-tailed *t*-test. Differences were considered significant when *P* value was less than 0.05.

Results

hNPCs can be genetically modified to stably express GDNF

Following isolation from the human fetal cortex, hNPCs were expanded as neurospheres *in vitro* and passaged weekly using a mechanical chopping technique that permits cells to remain as a three-dimensional structure [16]. hNPCs were expanded and banked until passage 26, at which time cells were infected with a clinical-grade lentivirus encoding GDNF to generate $hNPC^{GDNF}$. hNPCs differentiated mainly into GFAP-expressing astrocytes, with $hNPCs^{GDNF}$ showing stable GDNF expression in ~60% of the population at 5 weeks postinfection compared with noninfected hNPCs with no detectable GDNF expression (Fig. 1a and b).

Survival, differentiation, and sustained GDNF expression in long-term transplants

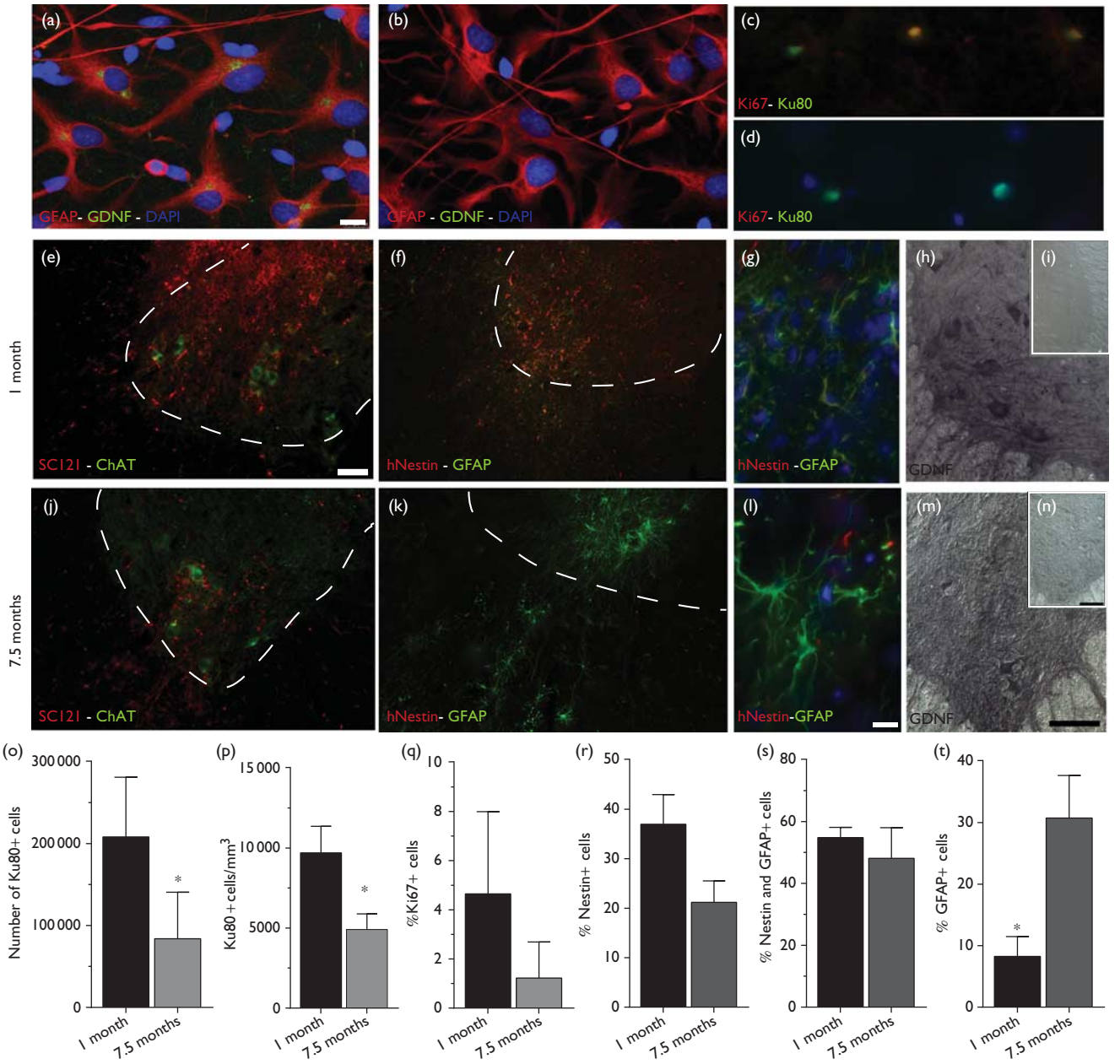
$hNPC^{GDNF}$ were transplanted directly into the spinal parenchyma of immunocompromised young-adult rats, specifically chosen to avoid the rejection of human cell xenografts and to allow sufficient time *in vivo* to characterize long-term cell transplants. Graft assessment using immunohistochemistry and cell counts showed that cell density (Fig. 1o) and cell numbers (Fig. 1p) decreased significantly by 2-fold and 2.5-fold, respectively, at 7.5 months post-transplantation compared with 1 month (cell density at 1 month, 9692 ± 744.7 cells/mm^3 and at 7.5 months, 4912 ± 4912 cells/mm^3, $P = 0.0043$; total cell numbers at 1 month, $207\,721 \pm 32\,780$ cells and at 7.5 months, $83\,463 \pm 33\,029$ cells, $P = 0.0472$). This lower cell density suggests decreased cell survival but also is consistent with cell migration away from the transplant site. All animals showed surviving transplants in the gray as well as the white matter of the targeted area and grafted cells could be observed in close proximity to choline acetyl transferase-positive motor neurons at both time points (Fig. 1e and j). There were only low levels of Ki67-positive proliferating cells observed at 1 and 7.5 months post-transplantation, and while there was no significant difference between time points (at 1 month, $4.6 \pm 1.5\%$ and at 7.5 months, $1.2 \pm 0.7\%$, $P = 0.1$) proliferation appeared to reduce over time (Fig. 1c, d, and q). Consequent to the low levels of Ki67 expression and cell proliferation, no long-term hNPC transplant ever showed signs of cell overgrowth, which attests to the safety of these cells.

At 1 month after grafting, the majority of cells expressed nestin or a combination of nestin and GFAP (nestin: at 1 month, $36.9 \pm 5.9\%$ and at 7.5 months, $21.2 \pm 4.3\%$, $P = 0.1$; nestin/GFAP: at 1 month, $54.8 \pm 3.3\%$ and at 7.5 months, $48.1 \pm 9.9\%$, $P = 0.55$) (Fig. 1f, g, r, and s), and GFAP-positive cells were observed within the core of the graft. At 7.5 months after grafting, cells migrated away from the transplant site and there was a significant increase in GFAP-positive cells (at 1 month, $8.2 \pm 3.3\%$ and at 7.5 months, $30.7 \pm 6.9\%$) (Fig. 1k, l, and t). Increased GFAP expression and morphology of grafted cells in long-term transplants suggest the increased differentiation of grafted cells into mature astrocytes. Importantly, grafted cells showed long-term GDNF expression and, in some instances, large spinal cord motor neurons stained for GDNF suggesting that the host cells were taking up the GDNF released by the $hNPC^{GDNF}$ transplants (Fig. 1h and m).

Discussion

We have previously shown that the injured environment in animal models of disease may provide a milieu that is more conducive to the survival of transplanted hNPCs compared with the intact environment in healthy controls [17,18]. However, animal models of disease

Fig. 1



Survival, differentiation, and sustained GDNF expression following long-term transplants of hNPCs. (a, b) Immunocytochemistry following *in vitro* differentiation shows $hNPC^{GDNF}$ (a) and control hNPCs (b) express the astrocyte marker GFAP (red) but only $hNPCs^{GDNF}$ express GDNF (green). (c, d) High magnification image of cells expressing Ku80 (green) and Ki67 (red) at 1 month (c) and 7.5 months (d) after transplantation. (e, j) Low magnification image of human cytoplasmic marker (SC121, red) and the motor neuron marker (ChAT, green) showing appropriate targeting of the transplanted cells to the ventral horn at 1 month (e) and 7.5 months (j) after transplantation. (f, k) Low and (g, l) high magnification images of cells expressing human-specific nestin (red) and human-specific GFAP (green) at 1 month (f, g) and 7.5 months (k, l) after transplantation. Note the increased GFAP expression and changes in morphology at 7.5 months. (h, i and m, n) GDNF expression is observed ipsilateral (h, m) but not contralateral (i, n) to the transplant at 1 month (h, i) and 7.5 months (m, n) after transplantation. Note the appearance of large cells stained for GDNF, which is presumably host motor neurons taking up GDNF secreted by the transplanted cells. (o, p) Stereological quantification of cells expressing Ku80 revealed a significant decrease in the number of grafted cells (o) and in cell density (p) at 1 month compared with 7.5 months after transplantation. (q) No significant difference in cell proliferation was observed at 1 month compared with 7.5 months after transplantation. (r–t) Quantification of cells expressing nestin and GFAP showed no difference in nestin expression (r, s) but a significant increase in GFAP-expressing cells at 7.5 months compared with 1 month after transplantation (t). Scale bars: (a)–(d), (g), (l), 10 μm; (e) and (f), (j) and (k), 75 μm; (h), (i), (m), (n), 100 μm. ChAT, choline acetyltransferase; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; hNPCs, human neural progenitor cells. *$P<0.05$.

may not work for long-term transplant assessment, as overt damage and disease progression can lead to animal death before the ideal time point for assessment of cell fate, function, and safety. Instead, young-adult healthy animals or immunodeficient animals (for xenografting without daily immunosuppression) can be used to extend the investigation of central nervous system (CNS) transplants to longer time periods. Here, we report the long-term survival of hNPCs in the spinal cord of athymic nude rats. Increased GFAP expression by the grafted cells indicates *in vivo* astrocyte differentiation. Importantly, even after long time periods, there was no significant increase in the proliferation of grafted cells and no overt signs of cell overgrowth.

There is a strong non-autonomous feature of motor neuron cell death in ALS where the glial cells surrounding motor neurons are dysfunctional leading subsequently to accelerated motor neuron death [19–21]. It has also been shown that the transplantation of astrocytes into murine models of ALS can ameliorate motor neuron cell death and improve function [22]. Furthermore, many transplantation-based therapeutic strategies in models of motor neuron disease or acute injury have attributed increased motor neuron survival and functional benefits to the production trophic factors by transplanted cells [23,24]. Finally, GDNF is a potent trophic factor for motor neurons [25,26] and when secreted from astrocytes has been shown to be neuroprotective in an acute model of motor neuron injury [27]. We have previously shown that hNPCs can be genetically engineered to express GDNF, that transplanted hNPCs^{GDNF} can mature into astrocytes and stably secrete GDNF in various rodent models of CNS injury [18,28] as well as in aged primates, and that hNPCs^{GDNF} [6] transplanted into the SOD1^{G93A} rat spinal cord can enhance motor neuron survival [10,14,15]. Here we augment the previous findings by showing that these same cells can survive and stably secrete GDNF for up to 7.5 months without forming tumors.

The safety of hNPCs combined with their differentiation into mature astrocytes and sustained neurotrophic factor delivery makes these cells a promising choice in cell-based therapeutic approaches for ALS and other CNS diseases where functional astrocytes and growth factor secretion may be of benefit.

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Conflicts of interest

There are no conflicts of interest.

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