Long-Term, Stable Differentiation of Human Embryonic Stem Cell-Derived Neural Precursors Grafted into the Adult Mammalian Neostriatum

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ABSTRACT

Stem cell grafts have been advocated as experimental treatments for neurological diseases by virtue of their ability to offer trophic support for injured neurons and, theoretically, to replace dead neurons. Human embryonic stem cells (HESCs) are a rich source of neural precursors (NPs) for grafting, but have been questioned for their tendency to form tumors. Here we studied the ability of HESC-derived NP grafts optimized for cell number and differentiation stage prior to transplantation, to survive and stably differentiate and integrate in the basal forebrain (neostriatum) of young adult nude rats over long periods of time (6 months). NPs were derived from adherent monolayer cultures of HESCs exposed to noggin. After transplantation, NPs showed a drastic reduction in mitotic activity and an avid differentiation into neurons that projected via major white matter tracts to a variety of forebrain targets. A third of NP-derived neurons expressed the basal forebrain-neostriatal marker dopamine-regulated and cyclic AMP-regulated phosphoprotein. Graft-derived neurons formed mature synapses with host postsynaptic structures, including dendrite shafts and spines. NPs inoculated in white matter tracts showed a tendency toward glial (primarily astrocytic) differentiation, whereas NPs inoculated in the ventricular epithelium persisted as nestin(+) precursors. Our findings demonstrate the long-term ability of noggin-derived human NPs to structurally integrate tumor-free into the mature mammalian forebrain, while maintaining some cell fate plasticity that is strongly influenced by particular central nervous system (CNS) niches.

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INTRODUCTION

Neural stem cells presently considered as cell therapies for neurological disorders are derived primarily from adult or fetal neural tissue or from in vitro differentiated embryonic stem cells [1–3]. Although both methods of derivation have advantages and disadvantages and new methodologies involving somatic cell derivation are being developed [4], the human embryonic stem cell (HESC) approach continues to provide a theoretically inexhaustible and very pliable source of cells [5]. The plasticity and differentiation potential of HESCs have been demonstrated in recent in vitro studies where these cells were induced to evolve into specific neuronal and glial lineages to become mesencephalic dopaminergic neurons [6–8], motor neurons [9–11], and oligodendrocytes [12]. The traditional ES cell differentiation paradigm involves the formation of embryoid bodies [13, 14] where cells evolve in a fashion similar to that in the embryonic gastrula; that is, they go through the three main embryonic germ layer stages. These cultures contain high admixtures of non-neuroepithelial cells [15], a condition that may be a disadvantage for neural...
replacement therapies that require more defined cell populations for transplantation. A new generation of protocols designed to avoid embryoid body formation has achieved a greater degree of in vitro commitment of HESCs to neuroepithelial fates [16, 17]. Further progress was made with the incorporation of protocols designed to mimic neural induction events that occur early in development [18], including those utilizing adherent monolayer conditions in which bone morphogenetic protein (BMP) signaling is blocked by noggin [19, 20]. These cultures allow for the homogenous exposure of HESCs to the neural lineage-promoting action of noggin and can reliably generate neural precursors (NPs) for further differentiation.

To test the generic potential of noggin-differentiated HESCs for therapeutic applications in the nervous system, we evaluated the in vivo outcomes of grafting these cells in the adult mammalian forebrain. In the present study, we demonstrate the consistent, controlled differentiation of HESCs to NPs using noggin without additional inducing agents or trophic factors and the ability of such derived NPs to integrate, to various degrees, into the adult mammalian forebrain. Within the course of several months explored in this study, HESC-derived NPs undergo substantial neuronal differentiation without apparent tumorigenesis, project axons to distant locations, and form structurally mature synapses in their innervation targets.

### Methods

#### Propagation and Neuroepithelial Differentiation of HESCs

The HESC line BG01, included in the NIH Human Embryonic Stem Cell Registry (http://stemcells.nih.gov/research/registry), was obtained from BresaGen, Inc., Athens, GA (http://www.novocell.com/) [5]. HESC colonies were grown on mitotically inactivated mouse embryonic fibroblasts (MEFs; 2 × 10^5 cells per 35-mm dish) prepared from E12 ICR mice (Taconic, Hudson, NY; http://www.taconic.com). Culturing conditions were according to company guidelines, except that higher concentrations of bFGF (10 ng/ml, Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and 15% knockout replacement serum (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) were used. Colonies were passaged every 4–7 days by manual dissection. For neural induction, days 4–6 HESC colonies growing on MEFs were treated with a medium composed of 50% fresh HESC medium (see above) and 50% Neurobasal (NB) medium (Invitro
can, supplemented with 100 ng/ml noggin (Sigma-Aldrich). Over the next 2 days, noggin concentration was gradually increased (day 2, 200 ng/ml; day 3, 250 ng/ml) and, on day 3, colonies were mechanically dislodged from the MEF layer and transferred to gelatin-coated tissue culture plates in NB complete medium. At 4 weeks, cultures were transferred to new gelatin-coated dishes (passage 1, P1) in NB complete medium with bFGF and noggin. NPs were passaged once again (P2) and then used for transplantation or were frozen. Details are provided in the supporting information data.

#### Monitoring Differentiation of HESCs In Vitro

**Quantitative Real-Time Polymerase Chain Reaction.**

Undifferentiated HESC colonies (days 4–5 after passaging) growing on MEFs were collected by mechanical dissection and placed into Buffer RLT (Qiagen, Valencia, CA, http://www1.qiagen.com). Total RNA was prepared according to manufacturer’s instructions. HESC colonies differentiating on gelatin-coated 35-mm dishes were lysed directly on the plates on days 7, 14, 21, and 28 and at P2 (1–2 plates per time point) using Buffer RLT. Total RNA was extracted from the lysates using an RNeasy kit (Qiagen) and was treated with DNase according to manufacturer’s protocol. Real-time polymerase chain reaction (RT-PCR) was performed essentially as described [2] (supporting information data).

**Immunocytochemistry.**

NP cultures were rinsed with phosphate-buffered saline and briefly fixed with 4% freshly depolymerized, neutral-buffered paraformaldehyde for 15 minutes. Cultures were processed for the immunocytochemical detection of neural stem cell and differentiation protein markers as per details described in supporting information data. Both single and multiple epitope immunocytochemistry (ICC) were performed and combined with DAPI counterstain. Total number of cells in 10 randomly selected fields was counted with the help of an automated cell-counting program (AxioVision; Carl Zeiss, Jena, Germany, http://www.zeiss.com).

**Immunophenotyping of NPs with Flow Cytometry.**

Cells were first dispersed into a uniform single-cell suspension using papain digestion protocol as described elsewhere [21]. The resulting mixture of cells was immunolabeled with the following cocktails of lineage-selective surface markers: rabbit IgG1 anti-human CD133 and mouse IgM anti-human CD15 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), mouse IgG1 anti-human CD29 (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com), and a mixture of tetanus toxin fragment C (TriTx)-mouse anti-TnTx IgG2b. Methodological details are included in supporting information data.

**Animals and Surgical Procedures**

Nude rats (strain CR: NIH-ncr; male; 6–8 weeks old; n = 22) were purchased from the National Cancer Institute. All surgical procedures conformed to protocols approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions and were carried out by using gas anesthesia (enflurane:oxygen:nitrous oxide = 1:33:66) and aseptic methods. NPs were injected in suspension (1.5 × 10^5 cells in 1 µl) into the neostriatum of animals mounted on a Kopf stereotactic unit (David Kopf Instruments, Tujunga, CA, http://www.kopf Instruments.com). Injections were made under microscopic guidance via pulled glass micropipettes controlled by a Nanoinjector device (World Precision Instruments, Sarasota, FL, http://www.wpi.com). Animals were killed with perfusion-fixation as described elsewhere [2] at 1.5 months (n = 8), 3 months (n = 7), and 6 months (n = 7).

The number of cells grafted was based on a pilot experiment designed to optimize integration of graft with host tissues and to monitor tumorigenesis as a function of graft size (see supporting information data, pilot experiment, and supporting information Fig. 1A–1E). In this pilot, we varied the number of cells (between 20 × 10^5 and 120 × 10^5) grafted in a single inoculation site into the neostriatum. Variance in the number of injected cells between 20 × 10^5 and 120 × 10^5 appeared to have little effect on the degree of tissue integration or the mitotic activity of grafts. Human-specific synaptophysin immunoreactivity was seen in small patches of grafted cells at 1.5 months, but increased substantially by 3 months and was seen throughout the graft at 6 months postgrafting (supporting information Fig. 2). Although the results of these pilot studies were reassuring with respect to general histopathology and tumorigenesis, the transient appearance of neuroepithelial phenotypes led us to decrease the number of cells in the graft to 15 × 10^5.

**Monitoring Differentiation of HESCs In Vivo: Histology and ICC**

The survival, growth, and phenotypic fate of HESC-derived NPs in vivo were assessed with immunoperoxidase or dual-label immunofluorescence. Tissues were prepared from animals perfused...
with 4% freshly depolymerized paraformaldehyde as described elsewhere [2]. Brain blocks were sectioned (30 μm) in the coronal or sagittal planes. Primary antibodies were used to disclose human (graft) versus rat (host) cell identity, mitotic activity, and neuronal and glial phenotype specification and included a number of well-characterized monoclonal antibodies and antisera (supporting information Table 1). Normal IgG from the species of origin of primary antibodies served as negative controls.

For immunoperoxidase staining, sections were processed with peroxidase-antiperoxidase or avidin-biotin-peroxidase [22]. Dual-label immunofluorescence was used to study NP mitotic activity and differentiation, combined immunoreactivity for human nuclear antigen (HNu) and that for a cell proliferation or differentiation marker, and was performed essentially as described elsewhere [2]. ICC controls were prepared by replacing primary antibodies with pre-immune serum from the same species of origin and were negative in all cases. To measure rates of differentiation of HESC-derived NPs, we used two representative sections through the middle of each graft and counted the total number of HNu(+) cells, and also the cells dually labeled with HNu and one of the chosen phenotypic markers on 10 randomly selected ×100 fields per case. The total number of HNu(+) cells and
RESULTS

Controlled Differentiation of HESCs to NPs In Vitro
HESC colonies were grown on a MEF layer supplemented with bFGF and differentiated to neuroepithelial fate with noggin (Fig. 1A, 1B). The pluripotency markers Oct3/4, SSEA4, Tra1-60, and Tra1-81 were amply expressed in these starting HESC colonies (Fig. 1C, 1D). After HESC colonies were dislodged from the MEF layer, they attached almost immediately to gelatinized plates and showed robust growth and flat appearance. Ten days after transfer, multiple early rosettes appeared at the center of the colonies. The cultures acquired three-dimensional growth at 15 days after transfer; at this time point, HESC colonies grew in the form of early rosettes (i.e., three-dimensional structures comprised of columnar epithelial cells) and proliferated vigorously. In 3 weeks, early rosette structures became less well defined and, by 1 month, most of them disappeared and cultures acquired two-dimensional neuroepithelial characteristics.

Differentiation of HESCs to NPs was monitored with RT-PCR, ICC, and flow cytometry (Figs. 1–3). RT-PCR data demonstrate that, with ongoing noggin treatment, there is a gradual upregulation of neural markers and a downregulation of pluripotency markers such as Oct3/4 (Figs. 1D, 2A), Nanog (Fig. 2A), and SSEA4 (data not shown). The stem cell marker Sox2 continues to be expressed in differentiating HESCs. Neural markers show gradual upregulation, with most of them rising between day 7 and day 21; further upregulation of neural markers is detected at passage 2 after the replating of NPs (Fig. 2A). The endodermal marker GATA-4 becomes undetectable by passage 2 (Fig. 2A). The neural crest marker Msi1 shows gradual upregulation (Fig. 2A), whereas two additional markers of mesoderm and endoderm (Brachyury and Pdx1, respectively) are absent during the differentiation process (data not shown). The expression of a panel of neurotrophic factors including the neurotrophins NGF and BDNF, GDNF, VEGF, and bFGF increases between day 14 and day 21 and then decreases or reaches a plateau (Fig. 2B). A representative panel of transcription factors conveying anterior, posterior, and dorsal-ventral position specification in the neural tube failed to demonstrate a predominant pattern in cultured NPs, despite some up- or downregulation of individual transcripts over time (Fig. 2C).

HESC-derived NPs were profiled by ICC immediately prior to transplantation (Fig. 3A–3E). More than 80% of the cells was immunoreactive for nestin (Fig. 3A). Seven percent of the cells expressed the class III β-tubulin epitope TU1, serving as a marker of immature neurons (Fig. 3B). Six percent of the cells expressed the astrocytic marker GFAP (Fig. 3C), whereas 10% of the cells expressed S100β, a marker of ependymal, radial glial, and early astrocytic cells (Fig. 3D). Multiple-antigen ICC confirmed further the predominant phenotypes of P2 NPs (Fig. 3E). For example, nestin was found to colocalize extensively with musashi-1 and vimentin, a pattern confirming the neural stemness of our NPs, whereas these cells were negative for pax6, an early marker of neuroepithelium. The neural stem cell marker CD15 (SSEA-1) was expressed by approximately 15% of NPs, whereas the common glial precursor marker A2B5 was present in less than 1% of NPs. Approximately 7% of the cells was mitotically active by Ki67 immunostaining (data not shown).

Immunophenotyping of P2 NPs by flow cytometry showed that less than 1% of the total NPs express the surface stem cell marker CD133 that denotes the presence of symmetrical division (Fig. 3F). In contrast, the great majority (approximately 79%) of NPs were positive for tetanus toxin (TnTx), a marker of neuronal progenitors [23, 24]. CD15−TnTx− cells comprised 16.80% of our NP population. A small subset (4.3%) of NPs was CD15+TnTx− (Fig. 3F-2) and 14.5% of these CD15+TnTx− NPs was CD29+ (Fig. 3F-3).

The above findings demonstrate the consistent derivation of multipotential NPs from HESCs by gradual differentiation. A combination of methodologies including ICC and immunophenotyping indicate that, at the time of grafting into brain, the majority of our NPs were at the stage of early neuronal progenitors [nestin(+)- and TnTx(+)-cells], whereas a sizeable one fifth or so of our NP population was at an earlier neural stem stage (CD15−TnTx− cells), capable of generating neurons and glial cells.

Survival and Proliferation of HESC-Derived NPs in the Neostriatum
To investigate the in vivo potential of HESC-derived NPs to integrate into the adult mammalian brain, we grafted P2 NPs into the neostriatum of young adult nude rats. HNu-immunostained material demonstrated the successful engraftment and excellent survival of NPs in this forebrain site (Fig. 4A–4C), with few scattered apoptotic profiles detected only in 1.5-month grafts. In some 1.5-month grafts, clusters of packed basophilic cells were present, but these were not observed at later time points. Because of the well-known propensity of HESC-derived grafts for tumorigenesis [25], we tested grafts for the expression of the nuclear proliferation marker Ki67 at 1.5, 3, and 6 months after transplantation (Fig. 4D, 4E). The Ki67 proliferation index within the grafts decreased from 8.3% ± 2.4% at 1.5 months (n = 8) to 4.2% ± 0.7% at 3 months (n = 7) and then to 1.2% ± 0.6% at 6 months (n = 7), suggesting a gradual exit from the cell cycle and differentiation of grafted NPs.

Consistent with a pattern of progressive maturation over time, the distinction of grafts from surrounding normal brain tissue, although pronounced at 1.5 months, gradually disappeared as the grafts assumed a more homogeneous appearance (supporting information Fig. 1). No teratomas [26], neural tumors [27], or clusters of Ki67 immunoreactive cells were observed within the grafts, findings that indicate the absence of tumorigenic proliferative masses.

Plasticity, Migration, and Neuronal Differentiation of HESC-Derived NPs
In the primary grafting location (neostriatum), the majority of NPs differentiated into TUJ-1-positive neurons (Fig. 5). Close to 50% (44.6% ± 3.3%) of HNu(+) cells were nestin-positive at 1.5 months, a rate reduced to approximately 10% (8.5% ± 1.4%) at 3 months and less than 1% (0.2% ± 0.1%) at 6 months after grafting. A large number of HNu(+) cells
Figure 2. Monitoring differentiation of HESCs in vitro by RT-PCR. Relative gene expression is shown as fold change in gene expression using the comparative Ct (ΔΔCt) method. Expression levels of genes in the initial HESC sample were used as standard (equal to 1) to which all other expression values were compared and expressed as fold changes. Bars corresponding to data from P2 NPs are shown in darker colors, to distinguish them from data derived from day 7 to day 28 HESC colonies that continuously differentiate within the same plates. (A): Expression of index pluripotency and neural markers in differentiating HESCs, measured by quantitative RT-PCR at days 7, 14, 21, 28, and 49 (P2). Stem cell markers are shown in yellow, early neural markers are in green, neuronal and glial markers are in gray, neural crest markers are in blue, and endodermal markers are in pink. The expression of mesodermal marker Brachyury was not detectable and, therefore, is not shown. (B): Expression of trophic factors including neurotrophins (NGF, BDNF, GDNF, VEGF, and bFGF) by differentiating HESCs at days 7, 14, 21, 28, and 49. (C): Expression of selected dorsal-ventral (blue), anterior (pink), and posterior (purple) markers by differentiating NPs at day 28 and P2. Several markers (Otx2, Emx2, Dlx2, and Nkx2.1) were not detectable and therefore are not shown. Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial-derived neurotrophic factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.
within the graft expressed doublecortin, a marker for migrating neuroblasts and early neurons [28, 29] (Fig. 5A). More than one half of HNu(+) cells were doublecortin(+) at 1.5 months (52.1% ± 3.4%) and 3 months (69.6% ± 5.3%); a similar percentage of HNu(+) cells also expressed the doublecortin-like kinase epitope DCAMKL-1 [72.0% ± 3.4% of HNu(+) cells] at 3 months after grafting. Doublecortin expression was reduced to less than 5% (4.7% ± 0.7%) of HNu(+) cells at 6 months after grafting (Fig. 5C). On the basis of a sharp rise in TUJ-1 colocalization of HNu(+) between 1.5 months (20.1% ± 3.9%) and 3 months (70.8% ± 6.8%) after grafting (Fig. 5B, 5C), we assume that neuronal fate was

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Figure 4. Transplantation strategy and survival of human NPs in rat neostriatum. (A): This is a composite sagittal map of individual striatal grafts at maximal inoculation size. Contours of each individual graft are shown in black. Contours of rat brain and the ventricular lining are in bold gray. The map shows a reliable coverage of a large section of anterior caudate-putamen with rare invasion into the adjacent globus pallidus. Major anatomical landmarks are depicted for orientation purposes (see abbreviations below). (B): This coronal section through the anterior septum shows a typical striatal graft of human NPs 3 months after inoculation. (C): This is an HNu-stained sagittal section through the middle of HESC-derived NP graft 3 months after grafting. The section was counterstained with cresyl violet. Most HNu(+) cells have large round nuclei consistent with mature neurons. The inset represents the magnification of framed area. (D, E): Mitotic activity within HESC-derived NP grafts as shown by dual staining for HNu and Ki67. (D): Mitotic profiles of NPs in grafts measured at 1.5, 3, and 6 months. Bars indicate group averages \pm SEM. Variance in mitotic profiles between the three cohorts was significant (ANOVA, \( p = .014 \)). Significant post hoc differences are indicated with brackets. (E): A representative area within a graft 3 months after transplantation, dually stained with HNu (red nuclear marker) and Ki67 (green nuclear marker). The three images illustrate the low rate of mitotic activity in HESC-derived grafts. The single double-stained nuclear profile (bottom) is indicated with an asterisk. Abbreviations: Acb, nucleus accumbens; AOD, anterior olfactory nucleus, dorsal; AOV, anterior olfactory nucleus, ventral; Cx, cortex; cc, corpus callosum; GP, globus pallidus; Hy, hypothalamus; STh, subthalamic nucleus; Str, corpus striatum (neostriatum); th, thalamus; VP, ventral pallidum. *, \( p < .05 \). Scale bars: (B, C): 100 \( \mu \)m (main panel), 10 \( \mu \)m (inset); (E): 10 \( \mu \)m.
established, for the majority of grafted NPs, prior to 3 months after grafting. Further increase of TUJ-1 expression between 3 and 6 months (to 86.6% ± 1.8%) was not significant.

Human-specific synaptophysin immunostaining was seen in islands of grafted cells at 1.5 months, but increased substantially by 3 months and was seen throughout the graft at 6 months after grafting (supporting information Fig. 2). There was no GAD or ChAT colocalization with HNu(+) profiles or human-specific synaptophysin(+) terminals in 6-month grafts. Only sparse colocalization of GluR2/3 and HNu was seen in grafts and there was no visible VGLUT1 or VGLUT2 immunoreactivity in human-specific synaptophysin(+) terminals. A number of markers of basal forebrain and basal ganglia neurons were expressed by differentiating NPs. DARPP-32 was not detected in 3-month grafts, but was expressed by nearly one third (30.1% ± 3.2%) of HNu(+) cells at 6 months after grafting (supporting information Fig. 3A–3C). The calcium-binding protein calretinin was expressed by less than 1% of HNu(+) cells at 3 months, but was present in 6.5% ± 1.6% of HNu(+) cells at 6 months after grafting (supporting information Fig. 3D, 3D'). Parvalbumin, another calcium-binding protein, was undetectable in 1.5- or 3-month grafts and was found in less than 1% of HNu(+) cells at 6 months after grafting.

In a few cases in which grafting inadvertently involved large white matter tracts of the forebrain, that is, the fornix, the internal capsule, or the genu of the corpus callosum, a majority of NPs had astroglial phenotypes as judged by confocal analysis of GFAP immunoreactivity in HNu(+) profiles and also with human-specific SC121 immunoreactivity (Fig. 6). In these cases, dual ICC for HNu and the oligodendrocyte markers O4 or RIP also revealed small numbers (less than 5%) of oligodendrocytes differentiating from grafted NPs.

Very few HNu(+) cells were found in parenchymal sites away from the graft. The only exception was the ventral forebrain pia which, in virtually all cases examined, was densely populated by HNu(+) bipolar cells; all these cells expressed doublecortin (supporting information Fig. 2), but were negative for nestin and did not display neuronal configurations; that is, they had no discernible axons by SC121 ICC. However, when HESC-derived NPs were sparsely deposited along the needle track and became part of the host ventricular epithelium, they all maintained a nestin-positive state months after the injection and none expressed doublecortin (supporting information Fig. 5).

In concert, our dual ICC experiments with graft-specific and differentiation markers indicate a predominantly neuronal differentiation of HESC-derived NPs in the neostriatal grafting sites. When cells were inoculated into white matter tracts, fate choice was astrocytic and, in some cases, oligodendrocytic. However, when human NPs were deposited in the ependymal region, they persisted in a neural stem cell state. The previous patterns demonstrate a fate-choice plasticity of grafted human NPs that may depend on host microenvironment. Parenchymal migration of those NPs was difficult to establish; pial neuroblast profiles consistently observed in all grafted cases could have arisen either from pial or via microvessel migration.

**Evidence for Circuit Integration of HESC-Derived NPs in the Rat Forebrain: Axons and Synapses**

Axonal projections from the graft were visualized as early as 1.5 months with doublecortin and DCAMKL-1 antibodies and at 3 and 6 months after grafting with human synaptophysin and human cytosol-specific SC121 antibodies (Fig. 7A, 7B). Human NF70(+) axons were evident only at 6 months after grafting (data not shown). These projections appeared as

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**Figure 5.** Neuronal differentiation of HESC-derived NPs in rat neostriatum. Photomicrographs represent cases of neuronal differentiation of HNu(+) cells by confocal microscopy from subjects prepared 3 months after grafting. (A): Dual ICC for HNu and doublecortin shows many HNu(+) cells that are also immunoreactive for doublecortin (DCX). (B): Double ICC for HNu and TUJ1 demonstrates the abundance of double-stained cells in the grafts. (C): Bar graphs depicting neuronal cell fate choice in striatal grafts of HESC-derived NPs at 1.5, 3, and 6 months represented by percentages of DCX-labeled (left panel) and TUJ1-labeled (right panel) HNu(+) cells. Bars indicate group averages ± SEM. Variance between the three cohorts was significant for both DCX (ANOVA, p = .0003) and TUJ1 (ANOVA, p = .0004). Significant post hoc differences are indicated with brackets. *, p<.05; **, p<.001. Scale bars: 10 μm.
dense bundles of axons traversing most white matter tracts
in the broad vicinity of the graft including the striatopallidal
pencils, stria terminalis, internal capsule, anterior commissure
(olfactory and temporal limbs), the forceps minor and body of
the corpus callosum, and furthermore, the cerebral peduncle
and callosal radiations.

Numerous human synaptophysin(+) boutons were found
in the surroundings of the graft as early as 3 months after
grafting; some of these structures made contact with other dif-
ferentiated NPs and these graft-derived cell-to-cell contacts
became even denser at 6 months (supporting information
Figs. 2, 3). Synaptophysin(+) boutons away from the graft
were evident at 6 months after grafting in layer I of frontal
cortex, the olfactory bulb (internal plexiform layer), anterior
olfactory nucleus, bed nucleus-central amygdala inserts in the
substantia innominata, the globus pallidus, substantia nigra-
pars reticulata, and the subthalamic nucleus (Fig. 7C).

Ultrastructural analysis of tissues from animals at 3 and
6 months after grafting stained for human synaptophysin
and SC121 in a pre-embedding fashion confirmed the presence
of synaptic structures originating from differentiated human NPs.
Human synaptophysin(+) terminals were especially dense
around the graft, in substantia innominata and in the bed nu-
cleus of stria terminalis. Labeled terminals were also evident
in frontal, motor, somatosensory, and auditory cortex and in
the olfactory bulb. Many labeled structures were en passant
swellings with round (38–50 nm in diameter) vesicles, sugges-
tive of synaptic vesicles, but did not exhibit pre- and postsyn-
paptic membrane specializations (not shown). Numerous la-
beled terminal swellings were also observed. In a few of
these terminals, synaptic vesicles and membrane specializa-
tions were visible at the same plane, allowing for their unam-
biguous classification as synapses (Fig. 7D–7F). In all these
synapses, the unlabeled postsynaptic elements had the

Figure 6. Astrogial differentiation of HESC-derived NPs in fornix (A, B), genu of corpus callosum (C), and internal capsule/globus pallidus
(C, D). Sections were processed for ICC with GFAP (A) or human cytosol-specific SC121 (B–D) antibodies. The section in (A) was processed
with immunofluorescence; sections in other panels were processed with immunoperoxidase. (A, B): In this case of spurious engraftment of human
NPs into the fornix bundle, the vast majority of HNu(+) or SC121(+) cells displayed astrocytic cytologies [both (A) and (B)] and GFAP expres-
sion (A). Insets in (A) represent a confocal microscopic analysis of the profile indicated by connecting lines; top inset is a traditional confocal
appearance of the index profile with a subsequent virtual resectioning at the x- and y-axis; bottom inset is a compressed z-stack image of the
index profile. Inset in (B) shows a low-power image of the fornix from which the main panel was derived. (C, D): In this case of a peripheral
involvement of the callosal genu (C) or globus pallidus (D) in the graft, there was a predominant differentiation of human NPs into astrocytes.
Insets in (C) and (D) are magnifications of framed areas in main panels. Scale bars: (A, C, D): 100 μm; (B): 10 μm.
structure of dendritic shafts or spines. Unlabeled postsynaptic structures were inferred to be of host (rat) origin due to their distance from the graft and the paucity of parenchymal migration of differentiated NPs. SC121 immunoreactivity was very similar in ultrastructural appearance to that of human synaptophysin.

In concert, differentiated grafts of human NPs show an early elaboration of axons that form bundles and traverse...
along established pathways in the host forebrain. Later grafts show an abundance of synaptophysin- or SC121-immunoreactive boutons in terminal fields; a number of these boutons demonstrate ultrastructural features of mature synapses.

**DISCUSSION**

**General Conclusions**

Our findings show an extensive neuronal differentiation of HESC-derived NPs and considerable structural integration into the adult rodent forebrain. The long time points used in this paper (up to 6 months) serve to show the stable, tumour-free differentiation that is key for the safety and therapeutic potential of HESC-derived NP grafts [30]. The long survival times used here also show the ongoing differentiation of these HESC-derived cells several months after grafting, that is, time frames that are unusual in stem cell transplantation studies [31], but may be necessary for establishing the full grafting potential of the slowly differentiating human cells. The number of NPs used for transplantation appears to be an important variable that, when optimized to the appropriate level (15 × 10^3 cells per animal), may eliminate neoplastic growth potential of grafted cells. Despite the advantages noted in pilot studies [34], the pluripotent markers Oct3/4 and Nanog are almost eliminated by day 28 of the noggin-based protocol due to the efficient neutralization of HESCs, thus drastically reducing the tumorigenic potential of grafted cells. Despite a predominant neuronal differentiation, such derived NPs were capable of making astroglial fate choices when inoculated into white matter tracks, a finding that supports the identity of a portion of these NPs as neural stem cells at the time of transplantation. Further support of their plastic nature at the time of transplantation is the arrest of these NPs in a nestin(+)-state in the case of inoculation in the ependymal region.

**Further Comments on Culturing Methodology**

The culture conditions used here to prepare NPs from HESCs and expand them prior to grafting were established in a previous report [20] in which we have also shown the karyotypic stability and lack of Neu9Gc contamination of these precursors. The present paper demonstrates further the advantage of the adherent monolayers of HESCs to high concentrations of noggin, which, by blocking BMP, arrests non-neural fate choices [33] and, further downstream, may also inhibit astroglial differentiation [34]. The pluripotent markers Oct3/4 and Nanog are almost eliminated by day 28 of the noggin-based protocol due to the efficient neutralization of HESCs, thus drastically reducing the tumorigenic potential of grafted cells. Despite a predominant neuronal differentiation, such derived NPs were capable of making astroglial fate choices when inoculated into white matter tracks, a finding that supports the identity of a portion of these NPs as neural stem cells at the time of transplantation. Further support of their plastic nature at the time of transplantation is the arrest of these NPs in a nestin(+)-state in the case of inoculation in the ependymal region.

Most NPs at the time of grafting were early neuronal progenitors, although at one fifth or so (the cell population exhibiting the CD15+ TnTx+ CD29- profile may give rise to radial glia and/or astrocytes). Optimizing between proliferation propensity and neural “stemness” of precursors is a challenging task that has been addressed and managed before [38]. A prompt reduction of mitotic index has been consistent across grafting experiments and is an especially encouraging finding of the present study. Reduction in mitoses was eventually also encountered in pilot grafts with much higher numbers of cells than the ones used in the main study, despite the tendency of such grafts to go through an initial period of neuroepithelial proliferation (supporting information Fig. 1). In contrast to forebrain transplantation, NP grafting into the brainstem and spinal cord showed a propensity for tumorigenesis and was not pursued further.

**Migration and Differentiation Choices of Human NPs**

On one hand, as we observed previously in the case of spinal cord grafts [2], the host microenvironment plays a decisive role in determining differentiation choices of grafted NPs. On the other hand, the large population of neuronal progenitors in our NP preparation is certainly consistent with the predominant neuronal differentiation of these NPs in the rat forebrain. Astrocytes present in mature grafts originated either in neural stem cells or in the small number of astroglial precursors present in the inoculum. Besides cell-intrinsic signals, such as POU, Notch, and bHLH, extrinsic molecular cues including noggin, BMPs, bFGF, EGF, LIF, and neurotrophins are likely to play key roles in promoting differentiation choices faithful to the cellular composition of each one of these diverse microenvironments [39–44]. The neuronal bias in differentiation of striatal grafts was evident in vivo with the prompt expression of doublecortin and then Tuj1 and synaptophysin by 3 months after grafting, although the expression of neurofilament markers and axonal elongation/pathfinding and elaboration of terminals for most forebrain targets would take another 3 months. This long time course of in vivo differentiation may be appropriate for the longer differentiation cycle of human NPs [45] and argues for the extension of survival times in human NP grafting experiments beyond the conventional 10–12 weeks. Despite advanced neuronal differentiation, including the late acquisition of DARPP-32 by one third of human NPs, these cells did not appear to achieve definitive neurotransmitter specialization, that is, become GABAergic neurons.

DARPP-32 is an early marker of lateral ganglionic eminence (LGE)-derived basal forebrain neurons, including medium spiny striatal projection neurons [46], and has been found in at least one other occasion in neurons differentiating from primate ES-derived progenitors grafted into striatum [47]. Expression of DARPP-32 in graft-derived neurons indicates that a substantial number of grafted NPs correctly acquired basal forebrain identity, yet failed to achieve the expected GABAergic phenotype of striatal projection neurons by 6 months after grafting. It is possible that whereas striatal progenitor cells acquire GABAergic and DARPP-32 identities under the influence of synchronized inductive signals in the developing LGE, in artificial systems combining initial in vitro differentiation followed by further maturation in the adult striatal environment NPs may acquire the one (i.e., DARPP-32) but not the other (i.e., GABA) differentiation marker [46, 48–52]. The importance of temporally and positionally appropriate inductive signaling is evident in studies using ex vivo derived NSCs as spinal cord grafts [1, 2], where spinal cord-derived NPs may be better able to undergo neuronal differentiation to GABAergic neurons compared to brain-
derived precursors [53]. Another possibility is that human NPs require more time in vivo to achieve full GABAergic differentiation.

**Axons, Synapses, and Structural Integration in the Host Forebrain**

SC121 ICC labels all axons projecting from differentiated NPs and shows that these axons take established pathways in their vicinity but do not grow through the gray matter; in doing so, these axons reach both the appropriate innervation targets of the striatum such as globus pallidus and subthalamic nucleus but also nonconstitutive targets such as neocortex. This pattern is consistent with previous findings that intact white matter tracts organize the axonal trajectories of transplanted developing [54, 55] and adult [56] neurons in a parallel molding fashion that is mediated, among other things, by a neurite-inhibitory effect of myelin [57].

The synaptic nature of terminal or en passant swellings labeled with human synaptophysin and SC121 was ascertained here by the presence of synaptic vesicles accumulating next to dense material on the cytoplasmic side of the terminal membrane [58], a synaptic cleft 20–30-nm wide, and an accumulation of dense cytoplasmic material on the postsynaptic membrane. The identity of such profiles as synapses between graft-derived and host neurons was based on either a combination of human synaptophysin immunoreactivity of the synapse and a postsynaptic structure remote from the graft or the combination of SC121 immunoreactivity of the synapse and an unlabeled postsynaptic structure in any location. Synaptic specializations between graft and host neurons were not visible at 3 months but were apparent at 6 months, that is, took several months before these human neurons established synaptic contacts with host postsynaptic structures.

**Relevance for Experimental Therapeutics**

Our findings demonstrate the generic ability of NPs derived from nongen-treated human HESCs and optimized for cell number and maturation stage, to survive and differentiate in the adult CNS without evident tumorigenesis. Despite a predominantly neuronal bias of grafted NPs based on the culture conditions used here, these cells maintain their cell fate plasticity and continue to respond to differentiation cues from particular CNS microenvironments. The substantial degree of neuronal differentiation of our striatal NP grafts raises the issue of their potential as replacement therapies for neurodegenerative diseases of the basal ganglia, such as Huntington’s disease. Such enthusiasm, however, should be tempered by the fact that these cells do not show complete differentiation into the main projection neurons of the striatum, that is, medium spiny neurons. With progress in our understanding of molecular cues specifying developmental fate choices in the basal ganglia-based forebrain, one can foresee modifications of the in vitro protocol reported here or ongoing treatment of these grafts in vivo. These additions will hopefully optimize the differentiation of human NPs and determine their potential as cell replacement therapies for diseases of the basal ganglia. In the mean time, their experimental therapeutic value lies primarily in their ability to make synaptic contacts with host neurons and, via these contacts, support injured host neurons with released trophic signals [41].

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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