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Induction of Adult Human Bone Marrow Mesenchymal Stromal Cells into Functional Astrocyte-Like Cells: Potential for Restorative Treatment in Parkinson's Disease

Merav Bahat-Stroomza · Yael Barhum · Yossef S. Levy · Olga Karpov · Shlomo Bulvik · Eldad Melamed · Daniel Offen

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Abstract Parkinson's disease (PD) is a neurodegenerative disorder with its motor phenomena due mostly to loss of dopamine-producing neurons in the substantia nigra. Pharmacological treatments aimed to increase the deficient dopaminergic neurotransmission are effective in ameliorating the cardinal symptoms, but none of these therapies is curative. It has been suggested that treatment with neurotrophic factors (NTFs) might protect and prevent death of the surviving dopaminergic neurons and induce proliferation of their axonal nerve terminals with reinnervations of the deafferented striatum. However, long-term delivery of such proteins into the CNS is problematic. We therefore aimed to differentiate ex vivo human bone marrow-derived mesenchymal stromal cells into astrocyte-like cells, capable of generating NTFs for future transplantation into basal ganglia of PD patients. Indeed, mesenchymal stromal cells treated with our novel astrocyte differentiation medium, present astrocyte-like morphology and express the astrocyte markers S100β, glutamine synthetase and glial fibrillary acidic protein. Moreover, these astrocyte-like cells produce and secrete significant amounts of glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and brain-derived neurotrophic factor as indicated by messenger RNA, real-time polymerase chain reaction, ELISA, and Western blot analyses. Such NTF-producing cells transplanted into the striatum of 6-hydroxydopamine-lesioned rats, a model of PD, produced a progressive reduction in the apomorphine-induced contralateral rotations as well as behavioral improvement in rotor-rod and the "sunflower seeds" eating motor tests. Histological assessments revealed that the engrafted cells survived and expressed astrocyte and human markers and acted to regenerate the damaged dopaminergic nerve terminal system. Findings indicate that our novel procedure to induce NTF-producing astrocyte-like cells derived from human bone marrow stromal cells might become a promising and feasible autologous transplantation strategy for PD.

Keywords Mesenchymal stromal cells · 6-Hydroxydopamine · Astrocytes · Parkinson's disease · Neurotrophic factors

M. Bahat-Stroomza · Y. Barhum · Y. S. Levy · E. Melamed · D. Offen (✉)
Laboratory of Neurosciences, Felsenstein Medical Research, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
e-mail: doffen@post.tau.ac.il

M. Bahat-Stroomza · Y. Barhum · Y. S. Levy · E. Melamed · D. Offen
Department of Neurology, Rabin Medical Center, Beilinson Campus, Petach Tikva, Israel

M. Bahat-Stroomza · Y. Barhum · Y. S. Levy · E. Melamed · D. Offen
Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

S. Bulvik
Laniado Medical Center, Netanya, Israel

O. Karpov
Brainstorm Cell Therapeutics Ltd, 12 Bazel St., Kiryat Arie, Petach Tikva, Israel

Introduction

Parkinson's disease (PD) is a common progressive neurological disorder caused primarily by degeneration of the

dopamine-producing neurons in the substantia nigra pars compacta. The cause is still largely undetermined. Therefore, most of the current pharmacological and surgical treatments for PD are targeted mainly at the disabling motor symptoms, often at the cost of serious side effects. In general, this approach does not modify the natural course of the illness or arrest or slow its progression. Therefore, researchers are turning to neuroregenerative strategies. These include gene therapy, wherein relevant genes are introduced via viral carriers or genetically modified cells in order to increase dopamine production (Chen et al. 2005a, b; Dass et al. 2006; Lu et al. 2005; Ryu et al. 2005), and the replacement of lost dopaminergic cells with similar cells harvested from mesencephalic tissues of human or pig fetuses (for review, see Levy et al. 2004). However, these methods are currently considered untenable. Besides the ethical concerns, clinical trials have raised questions regarding the survival of the engrafted cells and their ability to functionally integrate into the host brain, the possible induction of a local inflammatory reaction, and the clinical efficiency and risk of side effects, such as "off-medication" dyskinesia (Freed et al. 2001; Hagell et al. 2002). Nevertheless, given the survival of the engrafted cells and their production of dopamine, it is possible that the use of other cell sources for neuroregeneration or neuroprotection might be effective in PD.

An alternative approach is to restore surviving dopaminergic neurons by treatment with neurotrophic factors (NTFs), a family of proteins that regulates the survival, functional maintenance, and phenotypic development of neuronal cells (for review, see Levy et al. 2005). For instance, glial cell-line-derived neurotrophic factor (GDNF) is particularly important for the survival and function of dopaminergic neurons (Lin et al. 1993). The intrastriatal administration of GDNF in rats with 6-hydroxydopamine (6-OHDA)-induced lesions has been found to increase the density of the dopaminergic terminals and improve impaired motor functions (Rosenblad et al. 1998). Moreover, in a preliminary open-label study in patients with PD, direct long-term intrastriatal infusion of GDNF via pumps led to an increase in the length of "on-periods" with improvement in activities of daily living and reduced levadopa-related dyskinesia, without serious clinical side effects (Gill et al. 2003). However, a recent controlled trial using GDNF not only failed to demonstrate significant clinical benefit, it also raised concerns about potential adverse effects (Nutt et al. 2003). This finding might be explained by the mode of GDNF delivery into the central nervous system. Because of its molecular size, GDNF cannot cross the blood–brain barrier after systemic administration. Direct intrastriatal application from a single point source may lead to increased concentrations of GDNF in the vicinity of the catheter tip, so that the protein does not diffuse over larger distances to exert a widespread effect within the striatum (Kordower et al. 1999; Nutt et al. 2003). Thus, a more efficient mode of long-term intrastriatal delivery of GDNF or other trophic factors is needed.

Another, alternative approach to deliver NTF is to utilize stem cells. Although embryonic stem cells would probably serve as the ideal source because of their plasticity, ethical issues and the potential teratogenicity of these cells prompted us to focus our efforts on adult stem cells, namely mesenchymal stromal cells (MSC) derived from adult human bone marrow. Recent studies have suggested that MSC can be induced to differentiate into various cell types, such as hepatocytes and endothelial, cardiac muscle, skeletal muscle cells, and also neurons (Herzog et al. 2003).

The aim of our study was to determine if MSC can be induced to differentiate into functioning astrocyte cells. Astrocytes, a subtype of glial cell, playing a crucial role in the control of the homeostatic extracellular environment in the adult brain, are the main producers of NTFs, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), as well as GDNF (Müller et al. 1995). As part of their activity, astrocytes clear the brain of excess glutamate and dopamine, both of which might be toxic for dopaminergic neurons (Teismann et al. 2003). Culture of human MSC in our novel differentiation medium yielded cells that had an astrocyte-like morphology and expressed specific astrocyte markers. More importantly, these astrocyte-like cells secreted significant amounts of NTFs and provide neuroprotection against induced oxidative stress. When implanted into the striatum of a rat model of PD, they showed the ability to survive for a long term and were associated with an improvement in motor function, apparently due to an increase in the density of the dopaminergic fibers in the area of the lesion.

Materials and Methods

Isolation and Culture of Human MSC

MSC isolation and flow cytometry analysis were carried out as previously described (Blondheim et al. 2006). MSC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (SPN). The growth medium was replaced twice a week, and cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Differentiation of MSC into Astrocyte-Like Cells

MSC cells (1×10^6) were placed in a predifferentiation medium consisting of DMEM (SPN, L-glutamine) supplemented with 20 ng/ml human epidermal growth factor

(hEGF), 20 ng/ml human basic fibroblast growth factor (hbFGF; R&D Systems) and N2 supplement (5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, 100 μg/ml transferrin). After 72 h, the predifferentiation medium was replaced with DMEM (SPN, L-glutamine) supplemented with 1 mM dibutyryl cyclic AMP (dbcAMP), 0.5 mM isobutylmethylxanthine (IBMX; Sigma), 5 ng/ml human platelet derived growth factor (PDGF; PeproTech), 50 ng/ml human neuregulin 1-β1/HRG1-β1 EGF domain (R&D Systems) and 20 ng/ml hbFGF.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and stained with rabbit anti-glial fibrillary acidic protein (GFAP; 1:200, DAKO), rabbit anti-GDNF (1:100, Santa Cruz), rabbit anti-NGF (1:100, Santa Cruz), rabbit anti-BDNF (1:100, Santa Cruz), rabbit anti-glutamine synthetase (GS; 1:200, Sigma), or mouse anti-S100 beta (1:200, Sigma). Secondary antibodies were goat anti-rabbit Alexa-488 (1:200, Molecular Probes) and goat anti-mouse Alexa-568 (1:200, Molecular Probes). Nuclear DNA was stained by 4,6-diamino-2-phenylindole (DAPI; 1:1,000, Sigma). For GDNF staining, secondary antibodies were biotinylated goat anti-rabbit (1:200; Jackson Laboratories) and streptavidine-Alexa-488 (1:200, Molecular Probes).

Scanning Electron Microscopy (SEM)

The cells were fixed with 2.5% gluteraldehyde in phosphate buffer pH 7.2, washed in the same buffer, and post-fixed with 2% OsO_4. The third step of fixation was performed using a solution of tannic acid and guanidine hydrochloride. The triple-fixed cells were dehydrated in graded alcohol solutions. The alcohol was then exchanged for Freon 112 using graded Freon solutions. The cells were air-dried, gold-coated, and examined with a Jeol 840 scanning electron microscope.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated with TRIzol (Invitrogen). The RNA was reverse transcribed with random hexamers, and real-time quantitative PCR was performed on cDNA using the ABI Prism 7700 sequence detection system (Applied Biosystems). Sequence detector with Syber Green I served as the detection system.

The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-5′-CGACAGTCAGCCG CATCTT-3′ (forward) and 5′-CCAATACGACCAAA TCCGTTG-3′ (reverse); GDNF, 5′-TCAAATATGCCAGAGG ATTATCCTG-3′ (forward) and 5′-GCCATTTGTT TATC TGGTGACCTT-3′ (reverse); BDNF, 5′-AGCTCCGG GTTGGTATACTGG-3′ (forward) and 5′-CCTGGTGGA ACTTCTTTGCG-3′ (reverse); NGF, 5′-CA TGCTGGACCC AAGCTCA-3′ (forward) and 5′-GACAT TACGCTATGCA CCTCAGTG-3′ (reverse); GFAP, 5′-TAGAGGGCGAGGAG AACCG-3′ (forward) and 5′-GTGGCCTTCTGACACAG ACTTG-3′ (reverse); S100 beta, 5′-GGGTGAGACAAGGA AGAGGATG-3′ (forward) and 5′-GCTTGTGCTTG TCTCCCTCC-3′ (reverse); glutamine synthetase (GS), 5′-CGAAGGCCTGCAGAGACC-3′ (forward) and 5′-AGGG TATACTCCTGCTCCATGC-3′ (reverse). Data are presented as a ratio of mean threshold targeted gene expression to GAPDH. For each gene, the specificity of the PCR product was assessed by verifying a single peak on a melting curve analysis.

Measurement of Secreted NTFs

At the end of the differentiation process, the supernatant from the cells was measured for GDNF, NGF, and BDNF concentrations. The specific Emax ImmunoAssay (Promega) was performed according to the manufacturer's instructions. The absorbance at 450 nm was recorded on a Microplate Reader (BioRad Model 550). The results were calculated for one million cells results received from five donors.

Animal Model and Cell Transplantation

Male Sprague–Dawley rats (Harlan) weighing 220–280 g were treated with 6-OHDA hydrobromide using a Hamilton 10-μl syringe with a 26-gauge needle (12 μg/6 μl dissolved in ascorbate-saline). The material was injected into the left striatum at two sites (6 μl per site). The coordinates of the injections according to the Rat Stereotaxis Atlas (Paxinos and Watson 1985) were as follows: (1) anteroposterior (AP) +0.5 mm from bregma, mediolateral (ML) −2.5 mm, V −6.5 mm; and (2) AP −0.5 mm, ML −3.7 mm, V −6.0 mm. Six weeks later, 0.9% saline (n=6, control group) 5×10^5 vital astrocyte-differentiated cells/5 μl (experimental group, n=7) were stereotactically injected into the left striatum (2.5 μl per each site): (1) AP +1.0 mm, ML +3.0 mm, V −5.0 mm; and (2) AP +1.0 mm, ML +3.0 mm, V −4.1 mm. Animals were immunosuppressed by daily injections of cyclosporine (s.c. 10 mg/kg; Novartis) starting 24 h prior to surgery and continuing until sacrifice.

Animal maintenance and surgical procedures were conducted under the supervision of the Animal Care Committee of Rabin Medical Center and Tel Aviv University, Israel.

Measurement of Behavior Parameters

Induced Rotations At 3 and 5 weeks after intracerebral injection of 6-OHDA, the rats were injected subcutaneously

with the dopamine agonist apomorphine (Sigma, 0.15 mg/kg, dissolved in normal saline; Gonzalez and Kolb 2003), and contralateral turning was monitored 30 min later for 1 h using an automated rotation-measuring apparatus. Only rats with an average rotation rate of 5 turns per minute were included in the grafting experiments. The rotational behavior was measured again on days 60, 77, and 91 after transplantation.

Rotor-Rod Test At 110 days after grafting, the rats were examined for locomotor coordination, measured by the time of walking on a rolling rod (ROTOR-ROD™ System) adjusted to a speed of 16 rpm before falling off.

Sunflower Seeds Test This test, based on the study of Gonzalez and Kolb (2003), challenged the animals to open sunflower seeds for 5 min. Animal food was restricted to 45 mg/k/day for 5 days, and the number of seeds opened was measured on days 4 and 5 (115 and 116 days after grafting).

Immunohistochemistry

At 120 days after grafting, the animals were sacrificed, and the brains were perfused and removed as previously described (Bahat-Stroomza et al. 2005). Serial coronal sections of 20 μm thickness were cut with a microtome cryostat. The sections were rinsed extensively with PBS, placed in a citric acid buffer solution at pH 6.0, microwaved until boiling, and allowed to cool slowly until reaching room temperature. The tissues were then incubated with 5% normal goat serum for 1 h. Sections were subsequently incubated for 24 h with primary antibodies as described above, followed by mouse anti-human nuclear antibodies (hNu, 1:50; Chemicon) or mouse anti-tyrosine hydroxylase (TH; 1:200; Sigma). After washes, the sections were incubated with secondary antibodies (Alexa Fluor 568 1:200; Molecular Probes). For hNu staining, we used biotin goat anti-mouse (Zymed) and then streptavidin-Alexa Fluor-488 (1:200; Molecular Probes). Sections were coverslipped with fluorescence mounting medium (DAKO), and the slides were visualized with an Olympus BX52TF microscope (Olympus, Tokyo, Japan) equipped with a digital camera system attached to a Zeiss LSM410 confocal microscope. Images were processed using StudioLite™ software or Axiovison (Zeiss).

Fiber Density of Striatal Dopaminergic Nerve Terminals

The density of the TH fibers was calculated as the mean density of two representative areas of each slice following immunostaining with anti-TH antibodies. Image Pro-Plus software (Media Cybernetics, USA) was used. The analysis was performed on a total of 140 slides from three randomly selected rats in each group.

Statistical Analysis

In all experiments, Student's two-way *t* test was used for statistical analysis; $p<0.05$ was considered significant.

Results

Differentiated Human Bone Marrow MSC Demonstrate Astrocyte-Like Morphology and Astrocyte Markers

Before treatment, flowcytometry analysis of the bone marrow mononuclear cells was positive (>90%) for hematopoietic markers such as CD34 and CD45 and negative (<5%) for mesenchymal cell markers CD105, CD29, and CD73. However, after 6 weeks in culture (passage 5), we observed a marked reversal, and over 95% of the cells were positive for CD105, CD29, and CD73, while the hematopoietic markers completely disappeared. The differentiation capability of the MSC was evident by their successful induction to adipocytes as we previously described (Blondheim et al. 2006).

Differentiation was carried out in a two-step process. First, MSC were grown in the serum-free medium supplied with N2, bFGF, and bEGF for 72 h. Then, the cells were incubated for an additional 72 h with a serum-free medium containing FGF, cAMP, PDGF, IBMX, and neuregulin 1-β1 (as described in "Materials and Methods"). Whereas the cells grown in serum-free medium alone exhibited the typical flat fibroblast-like morphology (Fig. 1a,c), the cells further grown in the supplemented medium demonstrated satellite astrocyte-like morphology on both light microscopy and SEM (Fig. 1b,d). Immunostaining for astrocyte markers revealed that the morphological change was accompanied by the expression of S100 beta (24±2.4% of cells), GS and GFAP (30±1.6% of cells; Fig. 1e–g). On real-time PCR analysis, the transcripts of GS, S100 beta, and GFAP were higher by 2.7-, 2.4-, and 2.2-fold, respectively, in the astrocyte-differentiated cells than in the MSC (Fig. 1h).

Astrocyte-Differentiated Human MSC Express and Secrete Neurotrophic Factors

Astrocytes are known for their ability to secrete NTFs and growth factors. Real-time PCR evaluation of NTF transcript expression showed that messenger RNA (mRNA) transcripts of GDNF, NGF, and BDNF were higher by 10-, 24-, and 10-fold, respectively, in the astrocyte-differentiated

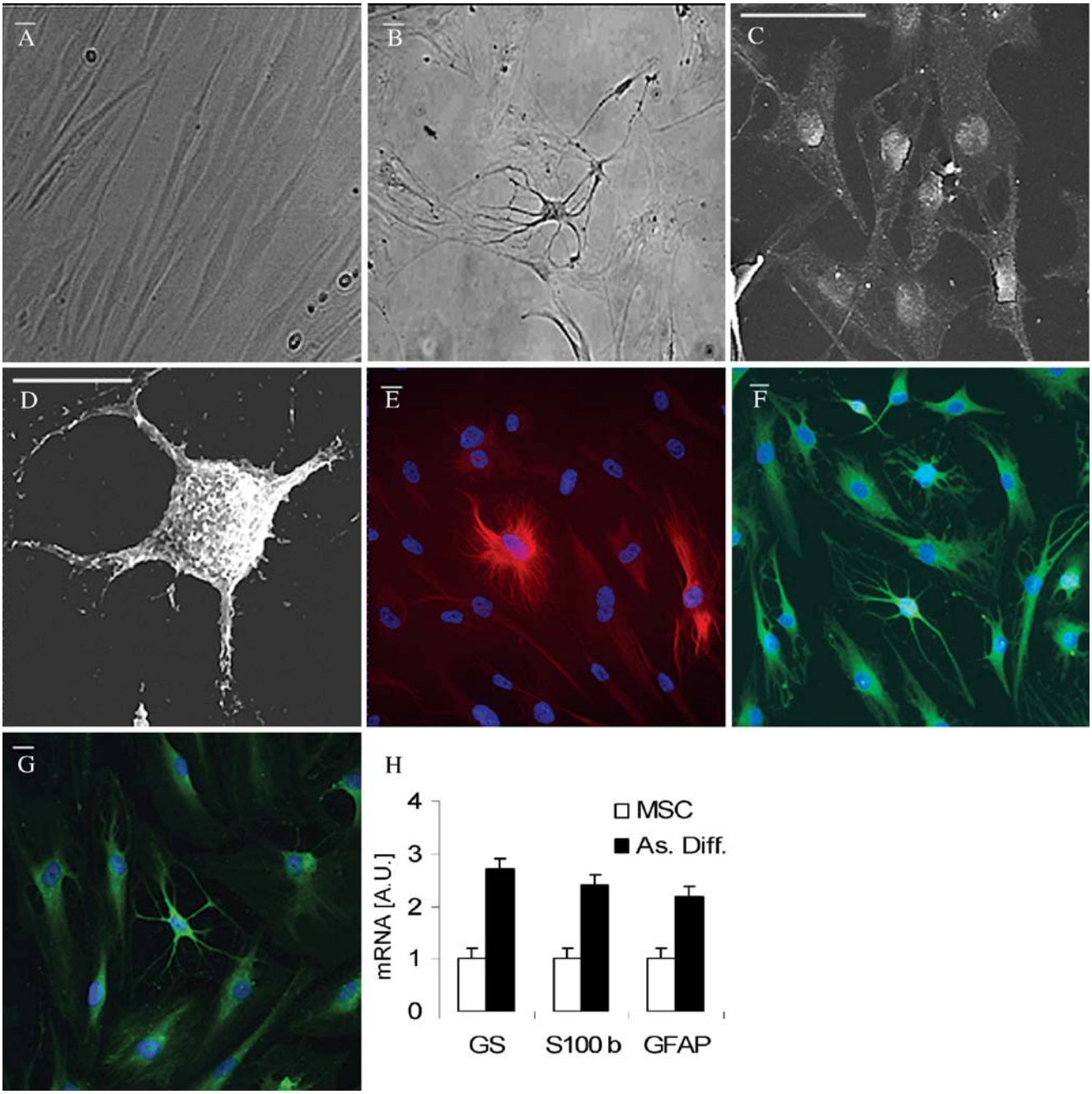


Figure 1 Differentiated MSC demonstrated astrocyte characteristics. Light microscopy images show the characteristic fibroblast-like morphology of MSC grown in serum-free medium (**a**) and the typical satellite-like morphology of astrocyte-differentiated human MSC following 48 h of differentiation (As. Diff.; **b**). Scanning electron microscopy (SEM) images demonstrate the flattened morphology of the MSC grown in serum-free medium (**c**) and the satellite-like morphology of astrocyte-differentiated MSC (**d**). Astrocyte-differentiated cells were stained with antibodies against S100 beta (**e**), glutamine synthetase (GS; **f**) and glial fibrillary acidic protein (GFAP, **g**). Nuclear DNA was stained with DAPI (*blue*; **e**, **f**, **g**). Scale bar—10 μM (**a**, **b**, **e**–**g**), **c** 100 μM; **d** 20 μM. Real- time PCR (**h**) was used to measure the mRNA of the astrocyte-differentiated cells for GS, S100 beta, and GFAP, and the findings were compared to human MSC grown in serum-free medium. *Y*-axis values represent the ratio of the specific mRNAs normalized to GAPDH in means ± SEM for three experiments. $p<0.05$ compared to serum-free treated MSC

cells than in the MSC (Fig. 2a). ELISA of the growth medium revealed that the cells secreted GDNF (329±164 pg/10^6 cells, $p<0.05$), BDNF (1,090±260 pg/10^6 cells, $p<0.01$) and NGF (260±76 pg/10^6 cells, $p<0.05$; Fig. 2b–d). These results were also confirmed by immunocytochemistry for NTF that revealed that the cells showing a change in morphology also positively stained for glial-derived neurotrophic factor (GDNF), nerve-derived neurotrophic factor

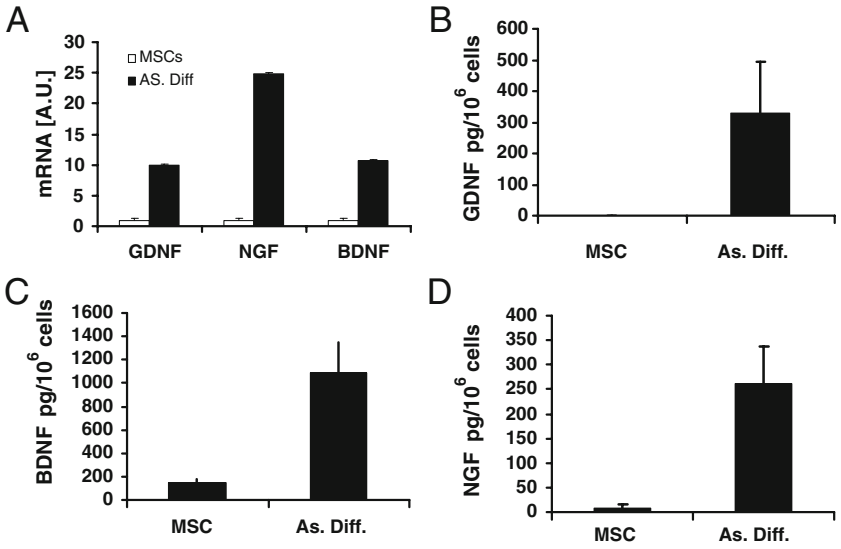


Figure 2 Astrocyte-differentiated MSC produce and secrete NTFs. Real-time PCR (**a**) of GDNF, NGF, and BDNF mRNA in astrocyte-differentiated MSC (*As. Diff.*) and MSC grown in serum-free media. RNA values represent the ratio of the specific mRNA normalized to GAPDH in means ± SEM for three experiments. ELISA measurement of GDNF (**b**), BDNF (**c**), and NGF (**d**) secretion from 1×10^6 MSC grown in serum-free medium or differentiation medium (*As. Diff.*). Results are presented as mean ± SEM of three independent experiments. Immunocytochemistry of the differentiated cells and MSC for glial-derived neurotrophic factor (*GDNF*), nerve-derived neurotrophic factor (*NGF*), brain-derived neurotrophic factor (*BDNF*; **f**, **h**, and **j** compared to **e**, **g**, and **i**) magnitude ×20

(NGF), and brain-derived neurotrophic factor (BDNF) as compared to control (Fig. 2f,h, and j).

Intrastriatal Transplantation of Differentiated Human MSC Reduces Rotational Behavior in a Rat PD Model

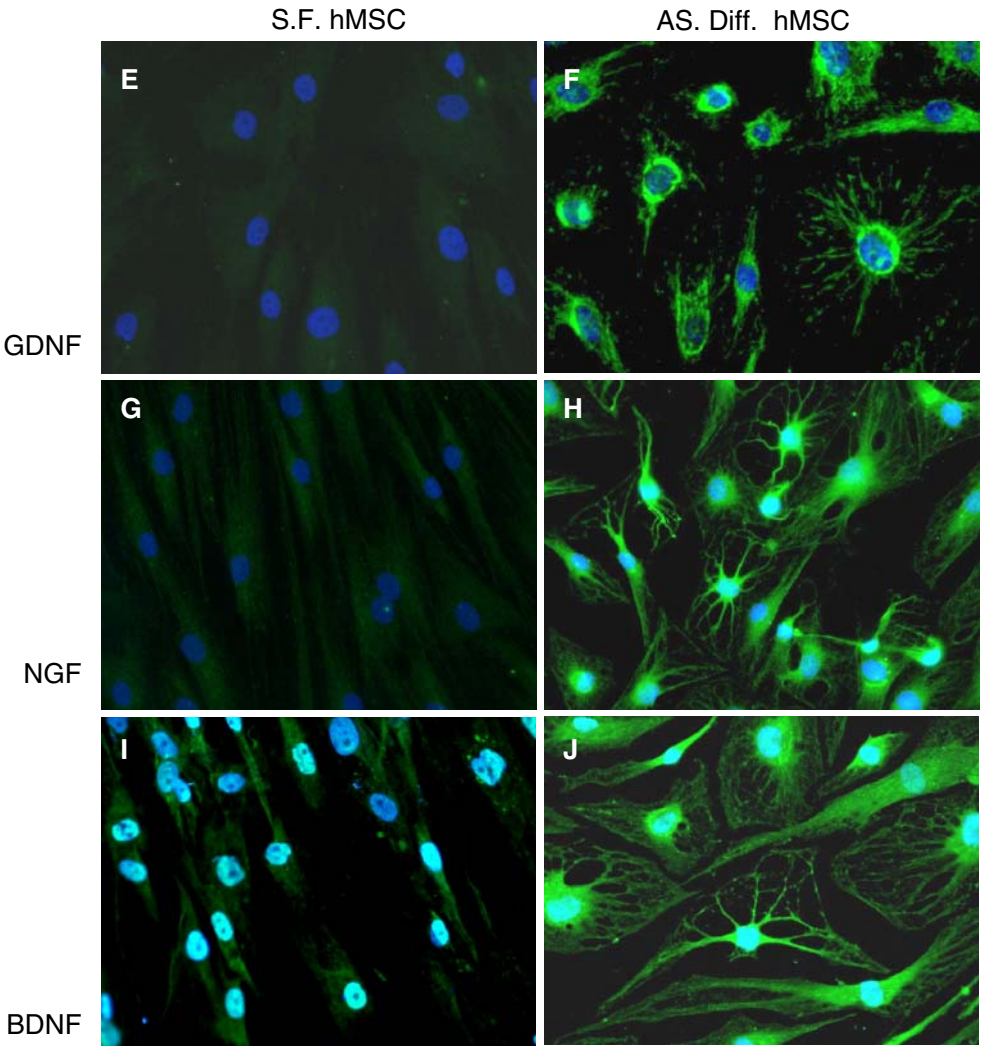
As NTFs are essential for the survival and function of dopaminergic neurons, we examined the behavioral effect of transplantation of the astrocyte-differentiated MSC in a rat model of PD. Six weeks after unilateral intrastriatal injection of 6-OHDA in the rats, saline or differentiated MSC (5×10^5) were engrafted into the striatum. The percentage of apomorphine-induced rotations was compared to the number of rotations of each rat before saline injection/cell transplantation at 60, 77, and 91 days. In the saline group, the rats demonstrated 86±24%, 98±20%, and 95±13% of the initial rotations, respectively. By contrast, in the cell-engrafted group, the rotations decreased to 60±14%, 40±13% ($p<0.05$), and 26±6% ($p<0.05$) of the initial rotations, respectively (Fig. 3a). We have used additional nonpharmacological tests to test the motor function. The sunflower seeds assay tests the delicate motor functions and the ability of the rat to use both paws to open seeds. We observed that the transplanted rats opened the seeds within 5 min more efficiently than the control (7.6±2 vs. 3.8±1.7 $p<0.05$, Fig. 3b). The rotor-rod assay that tests motor activity revealed that the transplanted rats were able to run for a longer period of time on the rod before falling off (32.8±4 s) as compared to the saline-injected rats (17.3±5 s; $p<0.05$, Fig. 3c).

Astrocyte-Differentiated Human MSC Survive Following Transplantation, Continue to Express Astrocyte Markers, and Enhance the Density of Dopaminergic Fibers in the Deafferented Striatum

We performed an immunohistological study to verify the presence, survival, and function of the transplanted cells. Coronal sections were stained with anti-human nuclear antibody to identify the human-originating transplanted cells. Confocal microscope images showed that the transplanted cells were located in regions close to the transplantation site in the striatum (Fig. 4a,b). Co-localization of hNu (green) and the GFAP astrocytic marker (red) was noted in this region (Fig. 4f,g), suggesting that the astrocyte-like identity was maintained for at least 120 days (time of sacrifice).

Previous experimental PD studies found that the administration of GDNF into the striatum increased the density of the dopaminergic nerve fibers (Kirik et al. 2001). To determine whether the transplanted astrocyte-like cells also affected the regeneration of the striatal dopaminergic fibers, striatal sections were stained with anti-TH antibodies. More than 20 sections per animal (three rats per group) for a total of 140 fields were evaluated. In the saline-injected control group, almost all the TH-positive fibers on the lesioned side were lost, with only 6.0±4% remaining compared to the unlesioned side (Fig. 5a,b). By contrast, in the rat transplanted group, 42.8±8% of the TH-positive fibers was demonstrated compared to the unlesioned side ($p<0.005$, Fig. 5c–e), indicating a proliferation of TH-positive

Fig. 2 (continued.)



nigrostriatal nerve terminals. Our observations indicate that the transplanted cells survived, kept their characteristics, and acted to regenerate the damaged dopaminergic nerve terminal system.

Discussion

In this study, we present in vitro and in vivo data emphasizing the potential of MSC to differentiate into cells that possess essential astrocyte functions. In the in vitro study, following our novel two-step protocol for astrocyte differentiation, the treated MSC exhibited the typical satellite-like astrocyte morphology accompanied by positive immunoreactivity to three major astrocyte markers: S100β, GFAP, and GS (Nakagawa and Schwartz 2004). Owing to their capacity for the production and release of NTFs such as GDNF, BDNF, and NGF, such cells are a promising option for the treatment of PD as well as a variety of other human neurodegenerative diseases. Following the in vitro study, we designed the in vivo experiment. Indeed, the therapeutic potential of astrocytes differentiated from human MSC was specifically supported in our in vivo study by the behavioral improvement seen in the 6-OHDA-lesioned rats after intrastriatal cell transplantation.

Astrocytes constitute a reservoir of trophic molecules that contribute to the maintenance of cellular homeostasis (Nakagawa and Schwartz 2004). Indeed, the functional activation of astrocytes has been reported following brain insults, such as trauma and ischemia, and in animal models of PD induced by 6-OHDA or MPTP (Nakagawa and Schwartz 2004). Although astrocytes were traditionally assumed to impede neuronal regeneration by forming glial scars, newer evidence indicates that reactive astrocytes that secrete NTFs may offer crucial benefits in functional recovery from brain injuries (Nakajima et al. 2001). NTFs are known to be particularly important for the survival and extension of the nigrostriatal dopaminergic neurons (Nakajima et al. 2001; Ransom 1991). Both patients with PD as well as animal models show a decrease in neurotrophins such as

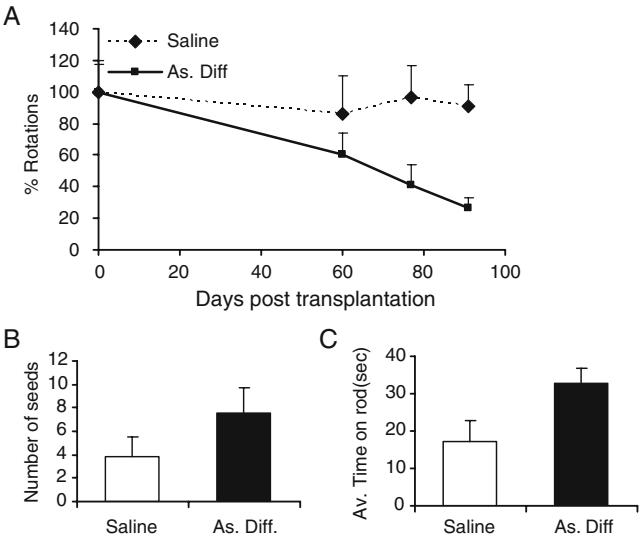


Figure 3 6-OHDA-lesioned rats after transplantation of astrocyte-differentiated human MSC demonstrate improved motor performance. Rats underwent transplantation with 5×10^5 astrocyte-differentiated (*As. Diff.*) cells by injection into the ipsilateral striatum 6 weeks after lesion. The apomorphine-induced (0.15 mg/kg, s.c.) rotation test (**a**) was performed and the average number of turns performed during 60 min was measured and compared with the baseline performance, measured twice before transplantation. The sunflower seeds test (**b**) was performed 116 days after grafting, and the number of sunflower seeds eaten in 5 min was measured. On the rotor-rod test (**c**), the length of time the animal could walk on the rod before falling off was measured. Findings are presented as mean ± SEM

BDNF, NGF, bFGF, and GDNF in the nigrostriatal dopaminergic regions (Chauhan et al. 2001; Chen et al. 2005a, b; Collier and Sortwell 1999; Nagatsu et al. 2000; Pekny and Nilsson 2005; Rudge et al. 1992; Yurek and Fletcher-Turner 2001). However, in some cases, following chemical injury (Chen et al. 2005a, b), the expression of several NTFs in the nigrostriatal pathway increased. This finding may point to a compensatory mechanism that protects the surviving neurons or stimulates their regeneration. Similarly, Nakagawa and Schwartz (2004) examined the expression of genes in reactive astrocytes of rats with 6-OHDA nigral lesions. They found that 1 week after lesioning, almost all astrocytes in the striatum were reactive, and the mRNA levels of GDNF, NGF, and bFGF were upregulated.

The effects of GDNF have been studied extensively in several models of 6-OHDA-induced PD. For example, implantation of GDNF-delivering microspheres to rats injected with 6-OHDA to the striatum increased neuron sprouting and preserved dopaminergic fibers (Gouhier et al. 2002). Similarly, in other PD models, GDNF delivery into the substantia nigra or the striatum by an adenoviral or lentiviral vector protected the nigral dopamine cells (Bjorklund et al. 2000). Moreover, bone marrow cells that were genetically engineered to express GDNF and then transplanted into MPTP-lesioned mice protected both the nigral cell body neurons as well as the striatal dopaminergic fibers (Park et al. 2001). Other NTFs, such as BDNF and NGF, were also shown to provide protection and to contribute to striatal fiber reinnervation in animal models of PD (Levy et al. 2004). Studies on the PC12 cell line showed that following exposure to 6-OHDA and MPTP, the exogenous application of NGF increased the level of antioxidant enzymes and heme oxygenase-1 and prevented apoptosis by inducing the anti-apoptotic gene-signaling pathways (Shimoke and Chiba 2001; Salinas et al. 2003).

Several researchers have shown that cultured MSC express low levels of astrocyte markers and NTFs such as BDNF, NGF, and GDNF (Crigler et al. 2005; Deng et al. 2006; Tondreau et al. 2004). In a previous study, we also found that cultured human MSC express an extensive assortment of neural genes and neural transcription factors at a generally low, but clearly detectable, level (Blondheim et al. 2006). These data indicate that MSC are predisposed to differentiate to neural lineages, but only after exposure to the proper conditions.

In the present study, we focused on the ability of astrocyte-differentiated MSC to produce and secrete NTFs. We therefore exposed the MSC to dbcAMP/IBMX, neuregulin 1-β1, and PDGF, all of which are associated with accelerated astrocyte maturation and differentiation (Arnhold et al. 2006; Pinkas-Kramarski et al. 1994). We established our protocol by screening for the proper combination of factors and culture medium that would yield the optimal NTF production. As indicated by real-time PCR and immunostaining, following differentiation, there was a marked increase in the astrocyte-specific markers, GFAP, S100β, and GS. Most significantly, high levels of secreted NTFs could be measured in the culture medium, indicating that the cells had undergone advanced maturation.

To further explore the possible therapeutic potential of these NTF-producing cells, they were transplanted into the striatum of 6-OHDA-lesioned rats. This model is most frequently used to study equivalent early and late stages of PD (Bjorklund et al. 2000). Similar to previous studies that tested NTFs, we performed a moderate lesion of the dopaminergic terminals and transplanted the cells at 6 weeks after lesioning, when the acute phase of degeneration of the nigral dopamine neurons is complete, as evidenced by the stability of the rotational behavior (Rosenblad et al. 1999). In our study, condition medium taken from the astrocyte–MSC culture demonstrate protection against H_2O_2-induced oxidative stress in neuroblastoma cell line (data not shown). Moreover, using the common rat model of PD, the cell-engrafted group showed a significant progressive reduction in apomorphine-induced rotational behavior as well as an improvement on other nonpharmacological motor tests. The decision to compare the effect of the differentiated cells to saline as control was based on our in vitro studies. They

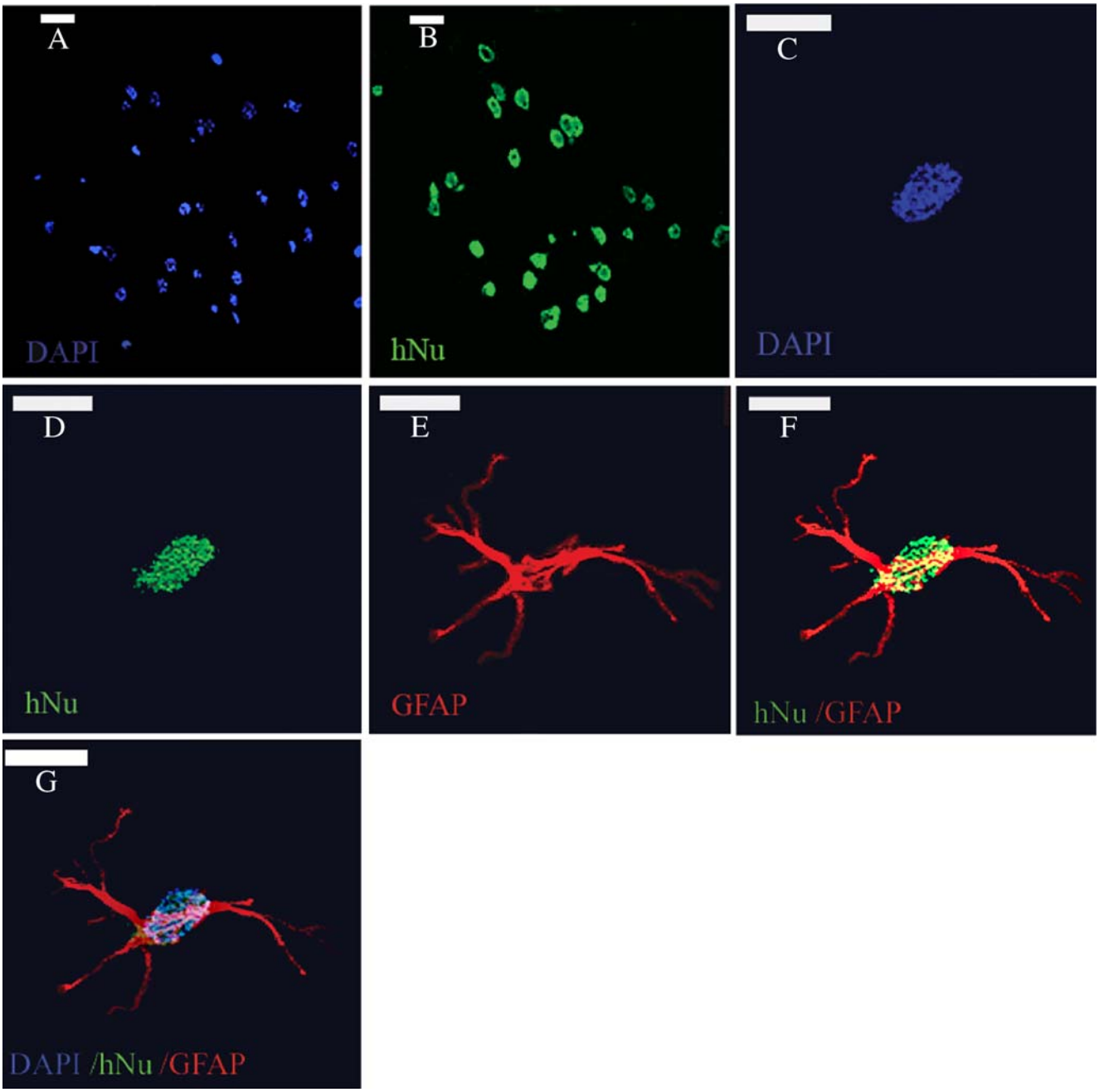


Figure 4 Transplanted cells survive and express the astrocyte marker GFAP. Confocal microscope images of the striatum show cells with positive staining for nuclear DNA (*DAPI*; **a**), human nuclear antibodies (*hNu*; **b**), indicating that the transplanted cells survived for the duration of the experiment. The astrocyte-differentiated human MSC were stained for nuclear DNA (*DAPI*, *blue*; **c**), hNu (green; **d**), and GFAP (*red*; **e**). The co-localization of DAPI with GFAP (**f**) and of hNu with GFAP (**g**) indicates both the human and the astrocyte nature of the cells in the engrafted rats

showed that the differentiated cells as compared to non-differentiated mesenchymal cells express and secrete high amounts of NTF and express astrocyte markers. In addition, we performed in vitro studies to evaluate the effect of the cells on a dopaminergic neuroblastoma cell line. We found that only the differentiated cells medium could protect this cell line against oxidative stress induced by 6-OHDA, whereas the media derived from nondifferentiated (MSC) cultures had no effect on cell survival (data not shown). This superior protection may be explained by the higher levels of NTFs shown to be secreted from the differentiated cells. The in vivo experiment was designed to emphasize the protection–restoration effect of the differentiated cells in 6-OHDA-induced partial lesion model with some residual surviving DA terminals. Protecting in a total dopaminergic lesion model, NTF cells are not expected to be useful and

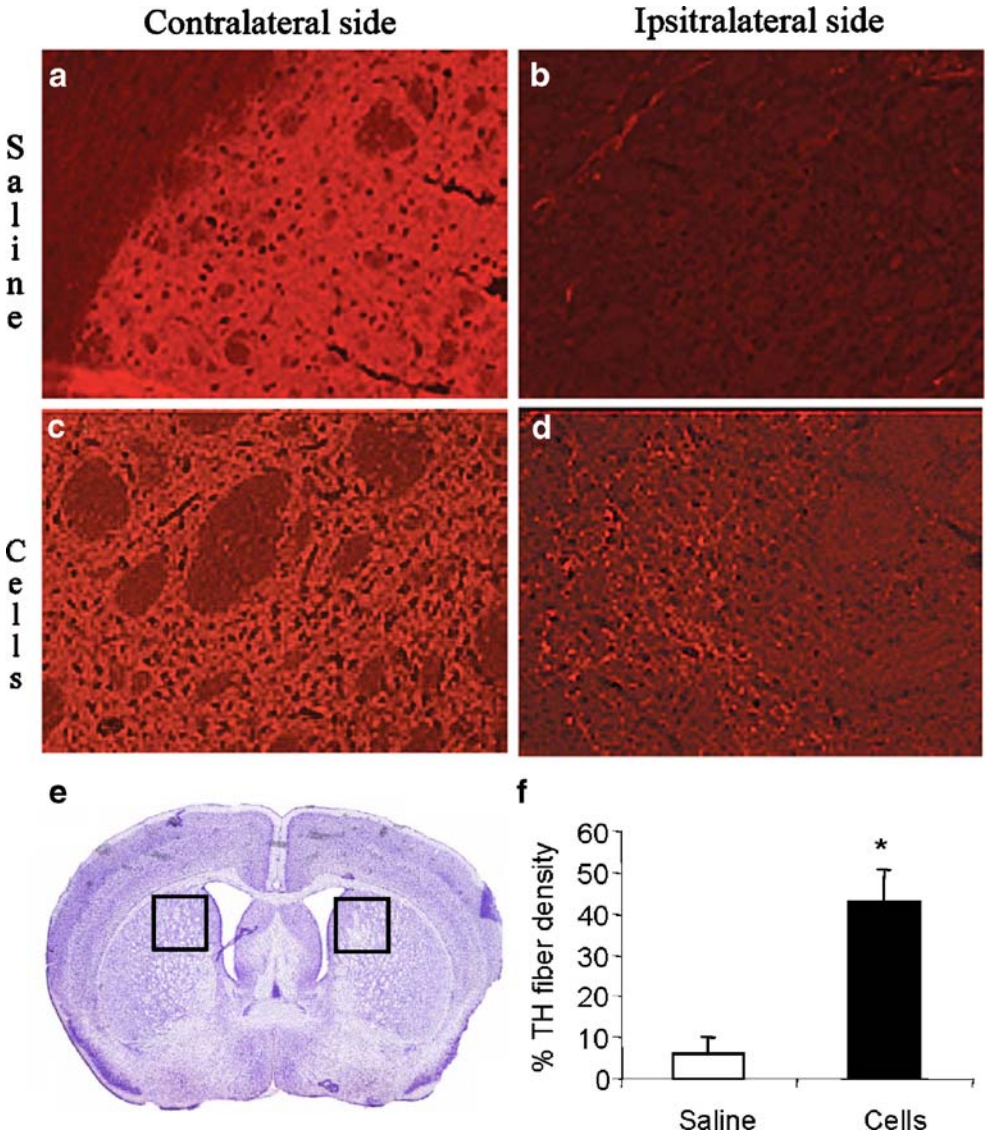


Figure 5 Enhancement of TH fiber density in the striatum following transplantation. Coronal sections of the striatum were immunostained for anti-TH antibodies, and the density of the TH-immunopositive fibers in the striatum was calculated. The *upper panels* show a representative figure of the intact (contralateral) side (**a**) compared to the 6-OHDA-lesioned (ipsilateral) side (**b**) in a rat injected with saline, and the *lower panels* show a representative figure of the intact (contralateral) side (**c**) and 6-OHDA-lesioned (ipsilateral) side (**d**) in a rat after cell engraftment. Note the coronal section from a rat brain atlas (**e**) showing the site at which TH fiber density was calculated. Adjacent (**f**) is the image analysis of the immunopositive fiber density. Results were obtained from computation of at least 20 sections per animal, three rats per group (total, 140 fields)

only replacement with dopaminergic cell might work as already shown in our previous study (Levy et al. 2008). However, the problem is that in a partial lesion, we expect some spontaneous recovery after a few weeks as was shown in several studies. Therefore, the proper control group should be 6-OHDA-lesioned rats injected with saline. We did not see any advantage for the use of other cell types as additional control groups based on our former experience in 6-OHDA animal models which failed to show efficacy when using fibroblasts or bone marrow-derived mesenchymal cells (Levy et al. 2008).

Histological studies indicated that the transplanted human cells, stained with anti-hNu antibodies, survived in the lesioned stratum until the end of the experiment (120 days). Moreover, the subpopulation of the cells maintained their immunoreactivity to the astrocyte marker GFAP, indicating the preservation of their astrocyte-like characteristics. The reduction in the apomorphine-induced rotations could have been due to sprouting of the nerve terminals of the remaining dopaminergic neurons in response to the NTFs produced and locally released by the engrafted human astrocyte-like cells. This assumption is supported by the marked increase in the density of the TH-immunoreactive fibers in the lesioned/engrafted striata compared to the saline-treated control group. Taken together, our data indicate that transplanted astrocyte-like cells, probably through their continuous NTF secretion, are capable of renovating and restoring the lost network of striatal dopaminergic nerve terminals in PD. To our knowledge, this is the first study to show that human-derived adult stromal cells may be differentiated *in vitro* into astrocyte-like cells, and improve impaired motor behavior in a rat model of PD. On the basis of our findings, we suggest that autotransplantation of a patient's own bone marrow-derived stromal cells transformed into functioning astrocyte-like cells might serve as a novel treatment of PD.

Such cells transplanted into multiple loci in the putamen and caudate nuclei might induce proliferation of dopaminergic nerve endings, leading to reinnervation of the denervated basal ganglia. Moreover, they may decelerate nigral neuronal loss and slow illness progression. The use of adult stem cells would bypass the ethical problems linked to embryonic cell sources. Furthermore, the use of the patient's owns cells for autotransplantation would circumvent the need for long-term administration of immunosuppressive anti-rejection agents.

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