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Regenerative effect of neural-induced human mesenchymal stromal cells in rat models of Parkinson's disease

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Background

Human bone marrow multipotent mesenchymal stromal cells (hMSC), because of their capacity of multipotency, may provide an unlimited cell source for cell replacement therapy. The purpose of this study was to assess the developmental potential of hMSC to replace the midbrain dopamine neurons selectively lost in Parkinson's disease.

Methods

Cells were isolated and characterized, then induced to differentiate toward the neural lineage. In vitro *analysis of neural differentiation was achieved using various methods to evaluate the expression of neural and dopaminergic genes and proteins. Neural-induced cells were then transplanted into the striata of hemi-Parkinsonian rats; animals were tested for rotational behavior and, after killing, immunohistochemistry was performed*

Results

Following differentiation, cells displayed neuronal morphology and were found to express neural genes and proteins. Furthermore, some of the cells exhibited gene and protein profiles typical of dopaminergic precursors. Finally, transplantation of neural-induced cells into the striatum of hemi-Parkinsonian rats resulted in improvement of their behavioral deficits, as determined by apomorphine-induced rotational behavior. The transplanted induced cells proved to be of superior benefit compared with the transplantation of naive hMSC. Immunohistochemical analysis of grafted brains revealed that abundant induced cells survived the grafts and some displayed dopaminergic traits.

Discussion

Our results demonstrate that induced neural hMSC may serve as a new cell source for the treatment of neurodegenerative diseases and have potential for broad application. These results encourage further developments of the possible use of hMSC in the treatment of Parkinson's disease.

Keywords

dopaminergic cells, mesenchymal stromal cells, Parkinson's disease, rat model of Parkinson's disease, transplantation.

Introduction

Regenerative cell-based therapy aims at grafting therapeutically relevant cells to impaired tissues and has been proposed for future therapies of intractable neurodegenerative disorders. Parkinson's disease (PD), characterized by progressive and selective loss of dopamine (DA) neurons in the midbrain substantia nigra, is a prime target for cell replacement therapy, with more than a decade of successful clinical experiences with fetal ventral mesencephalic cell transplantation in PD patients [1,2]. However, fetal cell transplantation has significant technical, ethical and practical limitations, partly because of limited availability and variable outcomes [2,3]. Stem cells, because of their self-renewal capacity and multilineage developmental potential, could be an ideal cell source for cell replacement therapy.

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It has been demonstrated that adult human bone marrow multipotent mesenchymal stromal cells (hMSC) are a subset of multipotential precursor non-hemopoietic cells from the bone marrow. They are notable for their ability to self-proliferate and differentiate along multiple lineages, including bone, cartilage, adipose and muscle cells [4,5]. Other researchers have shown that the cultured hMSC also have the potential to differentiate into neural cells [6–8]. As neural stem cells, they can migrate and integrate into the damaged brain [9,10]. However, it has yet to be demonstrated that these hMSC-derived neural cells have the capacity to function as neurons. hMSC can be easily isolated, cultured and expanded *in vitro* and their usage bears no ethical restriction. Moreover, hMSC enable the use of autologous transplantation, thus avoiding the risk of immune rejection.

We have previously derived highly enriched cultures of neural cells from hMSC [8,11,12]. These neural cells may serve as a platform for generating DA neurons to treat Parkinsonism. The major aim of this work was to investigate the ability of hMSC to differentiate *in vitro* toward functional dopaminergic neuronal-like cells. Neural and dopaminergic differentiation were characterized by the detection of neural markers and analysis of some level of neuronal function both *in vitro* and *in vivo*. Following neural induction, the majority of hMSC adopted a neuron-like morphology, expressed various neural genes and proteins, and acquired some functions of the dopaminergic neuron, such as production and release of DA precursor molecules. Finally, transplantation of these cells resulted in behavioral improvement of a rat model of PD. Our results firmly demonstrate that hMSC can differentiate along the neural pathways toward a dopaminergic functional phenotype. Moreover, we also provide evidence for an enhanced clinical benefit in PD rat models of the differentiated cells in comparison with naive hMSC.

Methods

Adult hMSC

Adult hMSC were collected from the iliac crest of 20 healthy human donors ranging in age from 20 to 70 years. The cells employed in this study were not pooled from multiple donors. The study was approved by the Tel-Aviv University and the Ministry of Health Helsinki Ethical Committee, and individual informed consent was obtained. The isolation, culture conditions, propagation and characterization of hMSC have been described in detail previously [8,12].

Induction and differentiation of dopaminergic neurons

hMSC were proliferated for at least 30 days from bone marrow isolation. To induce neural differentiation, hMSC (passage 2–7) were incubated for 24–72 h with stage 1 media, consisting of Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mm glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 12.5 U/mL nystatin (SPN; Biological Industries), basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), 10 ng/mL EGF, 30 μm docosahexaenoic acid (DHA; Sigma, St Louis, MO, USA) and N2 supplement (insulin 5 μg/mL, progesterone 20 nm, putrescine 100 μm, selenium 30 nm and transferrin 100 μg/mL; all from Sigma). Then the cells were incubated for 6–96 h with stage 2 media, composed of DMEM supplemented with 2 mm glutamine, SPN, N2 supplement, 200 μm butylated hydroxyanisole (BHA), 1 mm dibutyryl cyclic AMP (dbcAMP), 0.5mm 3-isobutyl-l-methyl-xanthine (IBMX), 100 μm ascorbic acid and 1–10 μm all-trans-retinoic acid (RA) (all from Sigma).

Transcript identification

Isolation and preparation of RNA

Total RNA was extracted from undifferentiated hMSC, hMSC incubated in neuronal differentiation medium for various lengths of time and human lymphocytes as a negative control, by using the guanidine isothiocyanate method [13]. Reverse transcription was carried out on 0.05-μg/μL mRNA samples using 5 U/μL enzyme SuperScript™ II RNase H–reverse transcriptase (RT) in a mixture containing 2 μm random primers (mostly hexamers), 10 mm dithiotheitol (DTT), 1 × buffer supplied by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA; http://www.invitrogen.com), 20 m dNTPs (TaKaRa Bio Europe, Gennevilliers, France; http://www.takarabioeurope.com) and 1 U/L RNase inhibitor (RNAguard; Amersham Biosciences, Amersham, UK; www.amersham.com). The reverse transcription reaction was performed at 25°C for 10 min, 42°C for 120 min, 70°C for 15 min and 95°C for 5 min.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were performed in a 20-μL final volume containing 2 μL reverse-transcribed RNA (cDNA), 0.5 μm sense and anti-sense primers, 1 × buffer supplied by the manufacturer, 225 m dNTPs and 1 U Taq DNA polymerase (TaKaRa). Primers were chosen from different exons to ensure that the PCR products represented the specific mRNA species and not genomic DNA. PCR conditions were optimized by varying the cycle numbers to determine a linear amplification range. cDNA underwent up to 35 cycles of amplification (1 min at 94°C, 1 min at 54–65°C and 1 min at 72°C) in PCR set PTC-100™ (MJ Research, Waltham, MA, USA). The PCR reaction was resolved on a 1% agarose gel. The bands were observed under UV light and photographed (VersaDoc™ model 1000 Imaging System; Bio-Rad Hercules, CA, www.biorad.com). Primer sequences and cycle numbers are provided in Table 1.

Northern blot analysis

Procedures were followed according to known protocols [14]. All reagents/materials were obtained from Sigma. Briefly, 10 μg total RNA from both untreated and differentiated hMSC were fractionated on 1% agarose containing 3% formaldehyde. RNA was then transferred to Duralon-UV™ membranes (Stratagene, Cedar Creek, TX, USA) by upward capillary transfer, cross-linked with 1200 mJ/cm^2 UV radiation (Hoefer Scientific Instruments, San Francisco, CA, USA). Probes for human neurite outgrowth-promoting protein 2 (NEGF2), neurofilament

Table 1. Primers used to detect neural transcripts in naive and neural-induced hMSC

Human gene NCBI no.	Forward	Reverse	Annealing temp (°C)	Size (bp)
CD90 (Thy-1) NM_006288	CTAGTGGACCAGAGCCTTCG	GCCCTCACACTTGACCAGTT	55	312
Catechol-O-methy ltransferase (COMT) NM_000754	TCGACAACAAATGGAGGACA	CAGAGGGGGATTGAAGTGAA	59	230
Dopamine β-hydroxylase (DBH) NM_000787	TCCAAGCTCCCAATATCCAG	TCGGGTTTCATCTTGGAGTC	56	223
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) NM_002046	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	55	194
Glypican 4 (GPC4) NM_001448	TGGGAAAGGCAAAAGCAG	CTCTCTGCATAACCAGGAAC	55	386
Necdin NM_002487	TAATCCCACTGCCCACTACC	TCTACCCCAACACACATCATC	55	394
Neurite outgrowth-promoting protein (NEGF) X55110	CCAAGACCAAAGCAAAGG	TTTATTGGGGGGAAAAAGTCAG	55	359
Neuron specific enolase (NSE) X13120	CTCAAGGGAGTCATCAAGGA	ATTGGCTGTGAACTTGGACC	55	356
Neurofilament heavy (NEFH) NM_021076	TGAACACAGAACGCTATGCGCTCAG	CACCTTTATGTGAGTGGACACAGAG	55	400
Neurofilament medium (NEFM) XM_005158	AAGCCACTCAGACCAGAATA	GCAGCGATTTCTATATCCAG	55	366
Retinoic acid receptor type α (RARA) NM_000964	AGCAGCAGTTCTGAAGAGATAGTGCC	CGTCAGCGTGTAGCTCTC	55	352
Ret proto-oncogene (RET) NM_000323.3	TGTGGAGACCCAAGACATCA	CCGAGACGATGAAGGAGAAG	55	233
Aldehyde dehydrogenase 1 *(*ALDH1) NM_000689	TGTTAGCTGATGCCGACTTG	TTCTTAGCCCGCTCAACACT	59	154
Dopamine receptor D2 (DRD2) NM_016574	AAGACCATGAGCCGTAGGAA	GTACAGGACAGGCGGGATGT	55	159
Dopamine transporter (DAT) NM_001044	GCTCACCCTGGGTATCGACAGCG	ATAGAACCAGGCCACTCCGATGGC	65, 55	253
GTP cyclohydrolase 1 (GCH1) NM_000161	TCTCAGATGTCCTAAACGATGCT	CAAGGACTTGCTTGTTAGGAAGA	59, 58, 57, 56	153
Monoamine oxidase B (MAOB) NM_000898	TCGACAACAAATGGAGGACA	CAGAGGGGGATTGAAGTGAA	58	237
Nuclear receptor related 1(Nurr1) NM_006186	AAAAGGCCGGAGAGGTCGTTTGCC	TGGGTTCCTTGAGCCCGTGTCTC	55	550

heavy (NF-H), neuron-specific enolase (NSE) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were prepared by specific primers (for primer sequences see the supplementary information at http://www.tau.ac.il/medicine/felsenstein/neurosci.html) in PCR apparatus. Probes were labeled with ^{32}P-dCTP (NEN Life Science Products, Boston, MA, USA) and the membranes exposed to a storage phosphor screen for various intervals. The hybridization signals were measured with a phosphorimager (Cyclone, Packard, Pangbourne, UK) and analyzed with OptiQuant™ software. Following autoradiography, membranes were stripped and rehybridized with a ^{32}P-dCTP-labeled probe for GAPDH, a housekeeping gene.

Quantitative real-time PCR

Quantitative fluorescent real-time PCR of the *NEGF* gene was performed to compare relative quantities of mRNA in differentiated cells using a Rotor-Gene 2000™ DNA sample analysis system (Corbett Research, Sydney, Australia). Real-time PCR reactions were carried out in a 10-μL final volume containing 1 μL reversed-transcribed RNA (cDNA), 0.5 μm sense and anti-sense primers (primer sequences are provided in table), 1 × buffer supplied by the manufacturer, 225 μ0 dNTPs, 0.05 U/μL *FB1-HS* DNA polymerase, 1 mm $MgCl_2$ and 300 × SYBR® Green I (Fisher Biotec, Perth, Australia). Samples were processed in triplicate. Standards were prepared using 10-fold dilutions of cDNA prepared from RT-PCR. Amplification of GAPDH was used for stimulated conditions for sample normalization. The amplification protocol was 95°C for 15 min followed by 55 cycles (95°C 15 s, 55°C 30 s, 72°C 10 s, 84°C 10 s). The relative quantity of NEGF2 gene expression to GAPDH was calculated by the standard curve method.

Protein detection

The Western blot analysis for protein-extracted hMSC and differentiated hMSC was prepared as described previously [8]. Primary antibodies (Ab) were employed in the following dilutions: mouse anti-actin (1:1000), mouse anti-NSE (1:1000), mouse anti-neuronal nuclear-specific antigen (NeuN; 1:1000), rabbit anti-tyrosine hydroxylase (TH; 1:6000 (all from Chemicon, Temecula, CA, USA) and mouse anti-NF-H 200 (1:1000; Sigma), followed by suitable goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary Ab (1:2000; Jackson Laboratories, West Grove, PA, USA). Proteins of interest were detected using the enhanced Super Signal® chemiluminescent detection kit (Pierce, Rockford, IL, USA).

Immunocytochemistry

hMSC were plated and treated in slide chambers (Nalge Nunc International, Napervilee, IL, USA) previously treated with 10 μg/mL human fibronectin (Chemicon). Cells were fixed with 4% paraformaldehyde and permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma) and 10% goat serum (to block non-specific binding sites; Biological Industries). The differentiated hMSC were stained with appropriate primary Ab: mouse anti-Tuj1 (β-Tubulin3; 1:400; Sigma), rabbit anti-α-Synuclein (1:500; Calbiochem, Darmstadt, Germany), mouse anti-microtubule associated protein2 (MAP2; 10 μg/mL; Zymed Lab, South San Francisco, CA USA) and rabbit anti-glial fibrillary acidic protein (GFAP, 1:80; Sigma), followed with suitable secondary Ab, goat anti-rabbit Cy3 or goat anti-mouse Cy2 (Jackson Laboratories). DNA-specific fluorescent dye 4,6-diamidino-2-phenylindole (DAPI; Sigma) counterstain was used to visualize cell nuclei. Cells were photographed using a fluorescence Olympus IX70-S8F2 microscope (Olympus, Tokyo, Japan) with a fluorescent light source (excitation wavelength 330–385 nm, barrier filter 420 nm) and a U-MNU filter cube, and/or with a digital camera system attached to a Zeiss LSM410 confocal microscope (Carl Zeiss, Göttingen, Germany).

Reverse-phase high-performance liquid chromatography

Reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with an electrochemical detector (ECD) was used to measure L-3,4-dihydroxyphenylalanine (Levodopa; DOPA). Briefly, DOPA levels were determined from samples that were collected from differentiation conditioned medium. Samples were stabilized with 0.1 m perchloric acid/metabisulfite (2 mg/mL; Sigma) and extracted by aluminum adsorption (Bioanalytical Systems, West Lafayette, IN, USA) [15]. An aliquot of filtrate was applied to HPLC/ECD (LC-4B and TL-5A; Bioanalytical Systems) equipped with a catecholamine C18 column (125 × 4.6 mm; Hichrom, Berkshire, UK) and with the electrode potential set at + 0.65 V versus the Ag/AgCl reference electrode. The mobile phase consisted of a monochloroacetate buffer (150 mm, pH 3) containing 10% methanol, 30 mg/L sodium l-octanesulfonate and

2 mm EDTA. The flow rate was 1.2 mL/min. DOPA was identified by retention time and validated by co-elution with cathecolamine standards under varying buffer conditions and detector settings.

6-Hydroxydopamine lesion animal model and cell transplantation

Male Sprague–Dawley rats (Harlan, Jerusalem, Israel), weighing 220–280 g, were unilaterally injected with 6-hydroxydopaminehydrobromide (6-OHDA; Sigma) (12 μg/6 μL dissolved in ascorbate-saline; Sigma), using a Hamilton 10-μL syringe with a 26-gauge needle, into the left medial forebrain bundle (MFB). The coordinates of the injections were AP −0.5, ML −3.7, DV −6 mm from bregma, based on the rat stereotaxis atlas [16]. Six weeks after the 6-OHDA lesion, the rats were divided into three groups. Rats in the control group ($n = 7$) were injected with 0.9% saline. The second rats group ($n = 7$) was injected with naive hMSC. Neural-induced hMSC were grafted into the rats of the third group ($n = 7$). Saline or 5×10^5 vital cells/5 μL was stereotactically injected into the left striatum, AP + 1.0, ML −3.0, DV −5.0 and −4.1 mm from bregma. Animals were immunosuppressed by daily injections of cyclosporine (s.c. 10 mg/kg; Novartis, Basel, Switzerland) starting 24 h prior to surgery and continued until they were killed. Surgical procedures were performed under the supervision of the Animal Care Committee at the Rabin Medical Center and Tel Aviv University.

Behavioral testing

Lesioned rats were evaluated for rotation behavior at 3, 5 and 7 weeks after the intracerebral injection of 6-OHDA. Contralateral turning was monitored for 1 h by an automated rotation-measuring apparatus after subcutaneous injection of the DA agonist apomorphine (0.15 mg/kg in saline; Sigma). Only rats with an average rotation rate of at least three turns/min were considered to be an established PD model and were used later for the grafting experiments ($n = 7$). The average number of rotations measured on weeks 5 and 7 after the lesion in each rat was considered as the baseline rotational behavior (100%). The rotational behavior was measured again at five time points post-transplantation (days 34, 64, 85, 99 and 125).

Immunohistochemistry

All animals were perfused a week after the last rotation measurement and the brains removed. Serial coronal sections of the brain were cut at 20-μm thickness on a microtome cryostat. Sections were rinsed extensively with PBS then placed in a citric acid buffer solution, pH 6.0, microwaved until boiling and allowed to cool slowly until reaching room temperature. Tissues were incubated with 5% normal goat serum for 1 h. Sections were subsequently incubated for 24 h with primary Ab: mouse anti-human nuclear (hNu; 1:50; Chemicon) or mouse anti-TH (1:200; Sigma), at 4°C. After washes, the sections were incubated with secondary Ab: Alexa Fluor 568 (1:200; Molecular Probes, Carlsbad, CA, USA). For hNu staining, biotin goat anti-mouse (1:200; Zymed Lab) and then streptavidine Alexa Fluor 488 (1:200; Molecular Probes) were used. Sections were coverslipped with fluorescence mounting medium (Dako, Glostrup, Denmark). Slides were visualized with an Olympus BX52TF microscope or a Zeiss LSM410 confocal microscope. Images were processed using StudioLite™ software (Olympus) or Axiovison (Zeiss).

Statistical analysis

Statistical significance for comparing groups was determined by using a two-tail unpaired Student's *t*-test. Tests were analyzed by Pearson correlation using one- or two-tail according to the assay. To assess behavior the one-way analysis of variance (ANOVA) test was employed.

Results

Characterization of hMSC

Human MSC were characterized for their cell-surface phenotype and mesenchymal differentiation capacity as described previously [8]. Briefly, cells were found to be positive for mesenchymal markers CD29, CD44, CD90 and CD105 (all > 95%) and negative for CD11b, CD19, CD34 and CD45. Adipogenic and osteogenic capacities were confirmed in accordance with the position paper regarding characterization of MSC [17].

hMSC expresses neuronal morphology and markers following induced differentiation

We induced neuronal differentiation in a two-step sequential process. To start with, hMSC were grown in a reduction serum medium supplied with DHA, N2, bFGF and EGF for 48–72 h. Then cells were incubated for an additional 6–96 h with a serum-free medium containing cAMP, BHA, RA and ascorbic acid. While cells grown in growth medium exhibited the typical flat

fibroblast-like morphology (Figure 1A), the induced cells had a spindle-shaped appearance with cell bodies and long branching processes typical of a neuronal appearance (Figure 1B).

We monitored the progression *in vitro* of hMSC differentiation toward neural fates at the level of gene expression using RT-PCR, Northern blot and real-time PCR analysis. RT-PCR of differentiation hMSC versus

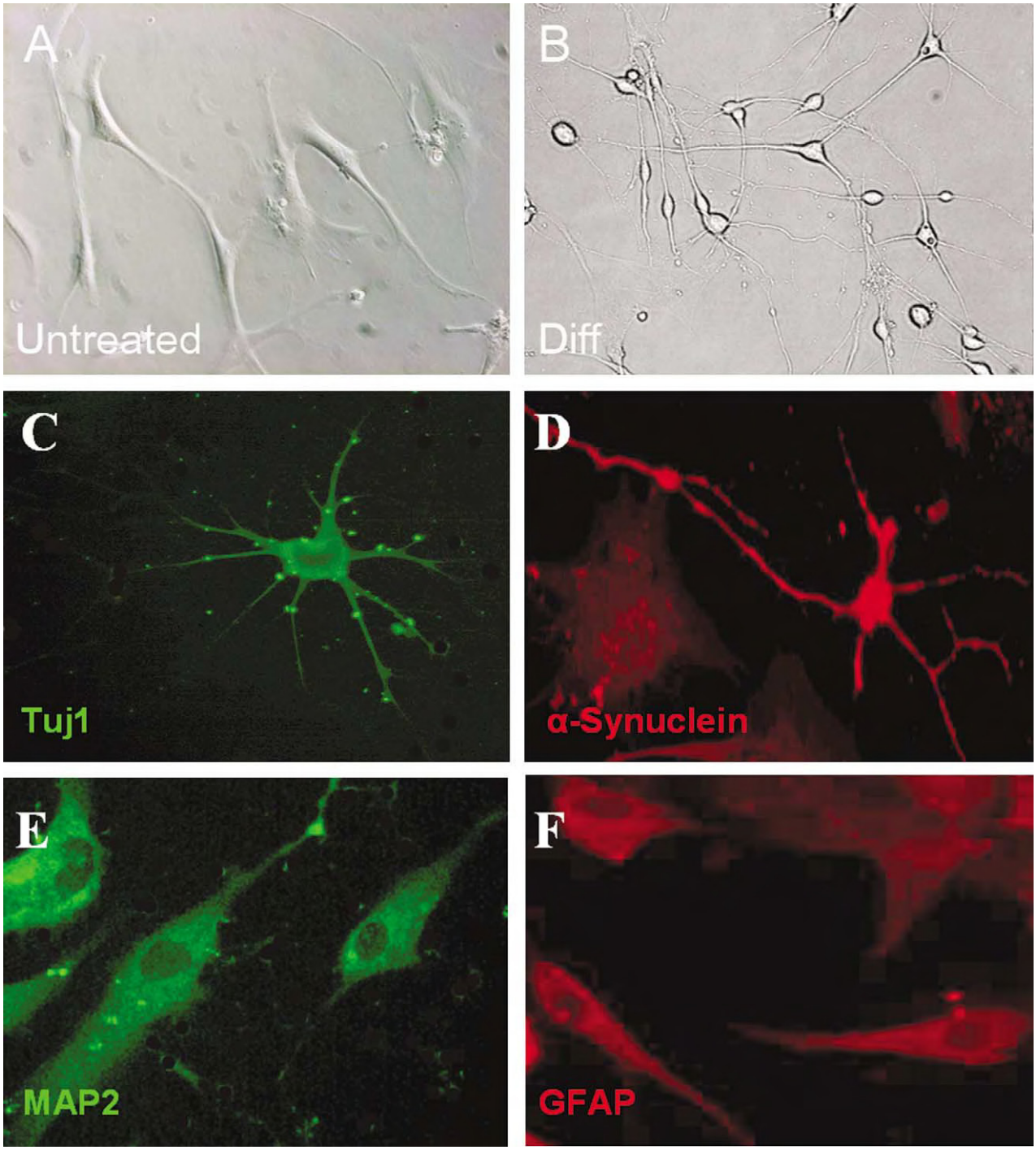

Figure 1. *Expression of neural markers following differentiation. Light microscopy of (A) naive hMSC and (B) neural-induced hMSC following 48-h differentiation. Immunofluorescence of neural-induced cells with Ab against (C) Tuj1, (D) α-Synuclein, (E) MAP2 and (F) GFAP. Original magnification × 20.*

Table 2. Neural genes and proteins expressed by naive and neural-induced hMSC

Gene name	Expression		
	Naive	Differentiation	Methods
Neural genes			
CD90	+	+ +	RT-PCR
Catechol-O-methyltransferase (COMT)	+	+ + +	RT-PCR
Glypican-4 (GPC4)	+	+ + +	RT-PCR
Necdin	−	+ + +	RT-PCR
Neurite growth-promoting factor 2 (NEGF-2)	+	+ + +	Northern blot
	+	+ + +	Real-time PCR
Neurofilament heavy (NF-H- 200 kDa)	+	+ + +	RT-PCR
	+	+ + +	Northern blot
	+	+ + +	Western blot
Neurofilament light (NF-L- 70 kDa)	−	+ + +	RT-PCR
Neurofilament medium (NF-M- 160 kDa)	+	+ + +	RT-PCR
Neuron-specific enolase (NSE)	+	+ +	RT-PCR
	+	+ +	Western blot
	+	+ +	Northern blot
Neuronal nuclei (NeuN)	+	+ + +	Western blot
Neurotrophic tyrosine kinase receptor type 2 (TRK-2)	+	+ +	RT-PCR
Ret proto-oncogene (RET)	−	+ + +	RT-PCR
Retinoic acid receptor type a (RARA)	+	+ + +	RT-PCR
Neurotrophic tyrosine kinase, receptor, type 2 (TRK-2)	+	+ +	RT-PCR
Tyrosine hydroxylase (TH)	+	+ +	Real-time PCR
GTP cyclohydrolase-1 (GTPCH1)	+	+	RT-PCR

−, No expression; +, very low expression; + +, intermediate expression; + + +, very high expression.
For RT-PCR the results are semi-quantitative.

naive hMSC demonstrated a significant increasing of neuronal transcripts CD90, catechol-o-methyltransferase (COMT), glipican-4, necdin, neurofilament medium (NF-160), neurofilament heavy (NF-200), NSE, Ret proto-oncogen (RET) and retinoic acid receptor α (RARA; results summarized in Table 2). Real-time quantitative PCR and Northern blot to the NEGF2 gene, an extracellular matrix-associated protein that enhances axonal growth in perinatal cerebral neurons, confirmed a 7-fold increase following differentiation (Table 2). In addition, Northern blot analysis for human NF-200 and NSE demonstrated that the expression of the genes also increased dramatically during neuronal differentiation and was time dependent, further increases correlating with the time–course of differentiation ($P = 0.031$, $r = 0.939$; $P = 0.051$, $r = 0.803$).

As for neural-associated protein expression, immunostaining for neural protein markers revealed that the morphologic change was accompanied by an expression of β-tubulin III, α-synuclein, MAP2 and GFAP (Figure 1C–F). In addition, Western blot analysis revealed that, during the course of 96 h of differentiation, the expression of NSE and Neu-N proteins increased markedly ($P = 0.04$, $r = 0.978$; $P = 0.03$, $r = 0.956$; 2- and 15-fold respectively; Table 1).

Effect of differentiation medium on the induction of dopaminergic precursors

hMSC were cultured in differentiation conditions for 12–72 h and RT-PCR was used to analyze the expression of dopaminergic transcription factor transcripts. We found that, prior to any induction, hMSC already expressed two of the transcripts that are essential for midbrain dopaminergic differentiation, Nurr1 and Pitx3 [18,19]. Moreover, we found that naive hMSC expressed very low level of TH, the rate-limiting enzyme in DA synthesis, and GTP cyclohydrolase1, the enzyme necessary for production of BH4, a cofactor of tyrosine hydroxylase (Table 2).

We identified that, following dopaminergic differentiation, the induced cells expressed transcripts of some of the machinery required for the biosynthesis, transport, synaptic packaging, release and re-uptake of DA. Among those were Aldh1, DA receptor D2 (DRD2), DA transporter (DAT) and monoamine oxidase B (MAO-B; which is partially responsible for breaking down dopamine; Figure 2A). No cells expressing the noradrenergic marker DA β-hydroxylase were observed in differentiated hMSC, suggesting that the differentiated cells were dopaminergic directed. In addition, the TH protein was significantly increased during the differentiation, as indicated by Western blot analysis ($P = 0.004$, $r = 0.979$; Figure 2B).

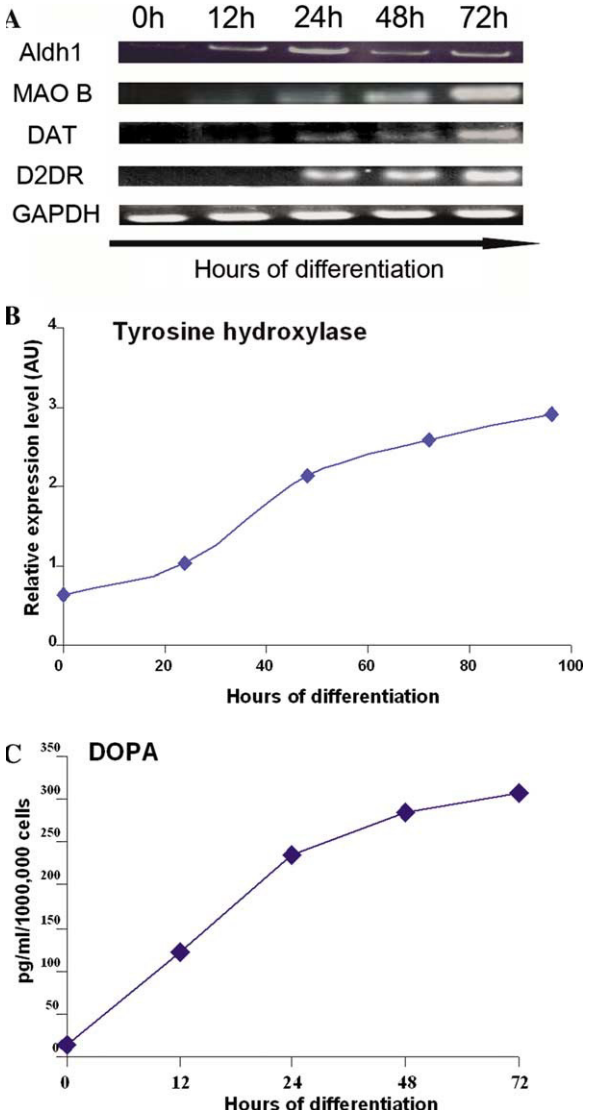

Figure 2. *Dopaminergic traits of neuronal-induced cells. (A) Gel electrophoresis of RT-PCR products of specific dopaminergic genes along the differentiation course (before differentiation), following 12, 24, 48 and 72 h of differentiation in differentiation medium. (B) Western blot analysis for relative expression of the TH protein following differentiation [results presented in arbitrary units (AU) in relation to β-actin]. (C) HPLC analysis of the DA precursor, DOPA, secreted to conditioned media following differentiation.*

To evaluate the functional dopaminergic capacity of the differentiated cells, reverse-phase HPLC was used to examine the ability of hMSC-derived neurons to release DA and its precursor DOPA. Consistent with the increase in number of dopaminergic neuron-like cells during differentiation, we found that the levels of the DA precursor, DOPA, in the supernatants were significantly increased during the time of differentiation up to 307 pg/mL/10^5cells ($P = 0.017$, $R = 0.904$; Figure 2C). No DA was detected employing this differentiation protocol. However, a previous study has described DA secretion following supplementation of the differentiation protocol described in this work with GDNF [12]. No DA was detected in naive MSC samples. The induced cells did not produce noradrenalin, which correlated with the lack of DA β-hydroxylase expression that was observed with RT-PCR (data not shown).

Functional recovery of Parkinsonian rats transplanted with neural-induced hMSC

We investigated whether the induced cells could elicit motor improvement in a PD rat model. 6-OHDA was injected into the left medial forebrain bundle and the animals were checked for baseline apomorphine-induced rotational behavior on weeks 5 and 7 after the lesion. Ten days after the baseline rotational behavior was determined, three groups of rats ($n = 7$) were injected in the lesioned striatum with either saline, naive hMSC or differentiated hMSC (5×10^5 cells). Over the next 4 months (days 34, 64, 85, 99 and 125 after transplantation), the apomorphine-induced rotations were compared with the baseline of each rat. We found that in the group injected with MSC-differentiated cells the apomorphine-induced rotations decreased to $80 \pm 4.7\%$ ($p < 0.1$) after 85 days, $71 \pm 8.9\%$ ($P < 0.1$) after 99 days and $58 \pm 10.8\%$ ($P < 0.05$) after 125 days. In contrast, both saline- and MSC-injected rats did not show a statistically significant reduction in the apomorphine-induced rotations (Figure 3A).

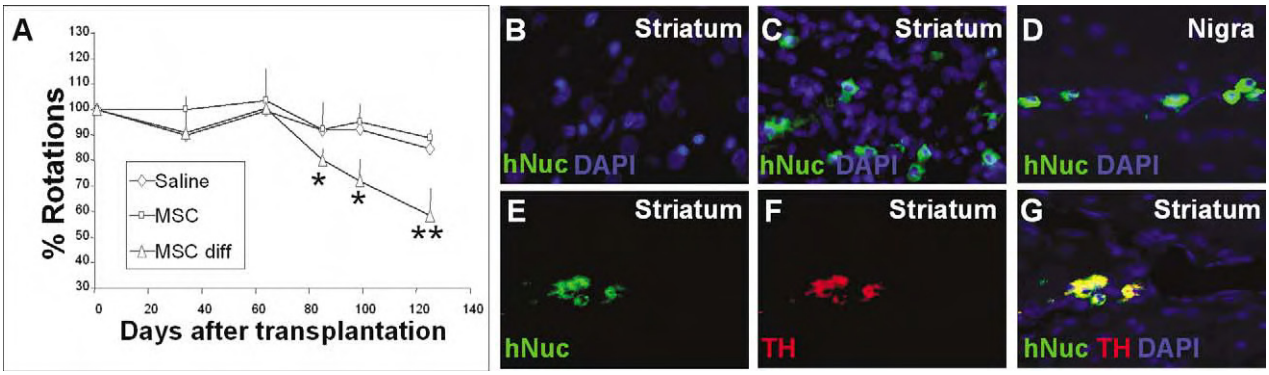

Figure 3. *Function and survival of neural-induced hMSC in brains of hemi-Parkinsonian rats. (A) Behavioral analysis: 6-OHDA lesioned rats were injected with saline (*n = 7*) or transplanted with naive (*n = 7*) or neural-induced hMSC (*n = 7*). Apomorphine-induced rotational behavior was assayed at five different time points following transplantation. The change in rotational behavior was calculated as a percentage of the baseline (see the Methods).* $^{*}P < 0.1$, $^{**}P < 0.05$. *(B–G) Immunohistochemical characterization of transplanted neural-induced hMSC. Grafted animals were killed and immunohistologic analysis performed. (B) Striatum representative of rat injected with saline, no hNu staining was observed. (C) Striatum of rats transplanted with differentiated cells stained for hNu. (D) Cells stained for hNu were located in the nigra ipsilateral to the lesion and transplantation site. (E–G) Some cells in the striatum stained positive for (E) hNu and (F) TH. (G) Merge of hNu and TH with DAPI counterstain. Original magnification × 20. Nuclear DNA was stained with DAPI.*

Survival and migration of induced hMSC in transplanted brains

We next explored the survival and function of the induced cells after transplantation into the left lesion striatum of Parkinsonian rats. The rats were killed for histopathologic analysis 1 week following the last behavior test. We used human-specific hNu Ab to identify human cells in transplanted rat brain sections. We found that a significant number of cells within the transplanted striatum was stained positive for the human nuclear antigen (Figure 3C). Furthermore, we detected some human cells in the nigra of the lesioned side, indicating cell migration toward the site of damage (Figure 3D). No human nuclei-positive cells were detected in control brains that were injected with saline (Figure 3B). In an effort to find dopaminergic-like cells, we stained the sections for TH. We found that some of the human cells detected in the striatum expressed TH (Figure 3D–F), indicating that the dopaminergic differentiation was sustained *in vivo* for more than 130 days.

Discussion

With this study we have described the beneficial effect of bone marrow-derived neural-induced cells transplanted into hemi-Parkinsonian rats. We have shown that neural induction of the MSC prior to transplantation proves to have a significant advantage over naive hMSC. The neural-induced hMSC were found to express elevated levels of genes associated with the neural lineage and were also found to express some proteins typical of neurons and glia. Moreover, we showed that the induced hMSC express specific dopaminergic genes and proteins and produce DOPA, the precursor of dopamine, suggesting a direction toward dopaminergic precursors. Above all, we have shown that transplantation of the induced cells into the brains of rat PD models resulted in a decrease in rotational behavior. The transplanted cells survived in the host brain for more then 130 days, migrated to the damaged nigra and expressed TH in the denervated striatum.

Recently, several studies have reported the successful engraftment, survival, migration and clinical improvement *in vivo* achieved upon introduction of animal MSC to the brains of animal models [20–23]. In parallel, reports have been made regarding successful attempts at generating neural-like cells from adult hMSC [24,25]. We therefore hypothesized that generation of neural-like cells, or even dopaminergic precursor-like cells, from hMSC prior to transplantation could facilitate the therapeutic effect of MSC. Therefore, we aimed at inducing *in vitro* differentiation and only then transplanting the induced cells to the 6-OHDA rat model.

In vitro differentiation was performed using a two-step induction protocol. For the first step, the cells were transferred to a lower serum containing media supplemented with N2, bFGF and EGF, which are known to

enrich neural precursor stem cells in culture [26]. The second step involved the withdrawal of serum and the addition of factors that were previously reported to be associated with neural differentiation and neuron survival. Among the factors present in the induction medium were intracellular cAMP-elevating compounds such as dibutyryl cyclic AMP and IBMX. cAMP has long been known to play a role in neuronal differentiation [27], enhance dopaminergic cell survival [28] and induce dopaminergic differentiation in mouse mesencephalic cultures [29]. Furthermore, the addition of cAMP has been reported to induce neurite outgrowth and expression of several neural markers in MSC in various works [30,31]. The differentiation medium was also supplemented with the anti-oxidant BHA, which was reported to prevent oxidative stress-induced apoptosis in primary neuron cultures [32]. Another differentiation agent included in the induction media was retinoic acid, which is known for its role in regulating neuronal differentiation and patterning of the developing nervous system [33]. This molecule was also reported to induce neuronal differentiation of MSC [6]. In an attempt to direct the cells toward a dopaminergic phenotype, we employed ascorbic acid in the induction media. Studies with embryonic stem cells and fetal mesencephalic cultures have shown the capacity of ascorbic acid to induce dopaminergic differentiation, up-regulate TH expression and improve dopaminergic cell survival [34,35].

The mechanism by which neural differentiation of MSC occurs is still unclear. However, a few recent reports suggest that the activation of cAMP-dependant mechanisms plays a significant role in the changes observed in cell morphology, up-regulation of neural genes and expression of neural proteins [36,37]. We have also shown in previous work that hMSC already expresses an array of neural genes and proteins prior to any induction [8]. Among those neural genes are the 'proneural' genes that belong to basic–helix–loop–helix (bHLH) family and are crucial for stem cell neuronal differentiation [38]. As few studies have described bHLH inducing neuronal differentiation as a result of cAMP pathway activation [39,40], we therefore based our differentiation media on the combination of cAMP analog (dibuturyl cAMP) and phosphodiesterase inhibitor IBMX, to enable a sufficient effect of increased intracellular cAMP levels.

We measured the gene expression level of various genes typical of neural cells by RT-PCR, Northern blot and real-time PCR. We found that many genes that are expressed at a basal level in naive hMSC are further up-regulated following induction. At the protein level, we found that, following induction, some of the cells highly expressed Tuj1, a neural progenitor marker, α-synuclein and MAP2, specific neuronal markers, and also GFAP, which was reported to be expressed in neural stem cells as well as in glial cells [41,42].

As our aim was obtaining dopaminergic-like cells, we screened the induced cells for expression of dopaminergic genes and proteins. As previously reported by our group [8], we observed a significant expression level of Nurr1, which is known to play a key role in dopaminergic differentiation, prior to any induction. The expression of Nurr1 may account for the basal level of expression of TH in MSC, which was also previously reported by our laboratory [8,12]. We also found a low basal level of expression of other transcription factors crucial to dopaminergic differentiation, such as Pitx3 and En1, in MSC before induction. However, following differentiation the differentiated cells expressed specific dopaminergic transcripts that were not observed prior to incubation in the induction medium. Among them were Aldh1, a marker of dopaminergic progenitors, MAO-B, which is responsible for DA degradation, DRD2 and DAT. Regarding TH, we found its low basal level of protein expression to be up-regulated along the course of differentiation. This result correlated with the increase in DOPA levels found in the conditioned media. At no time point did we detect noradrenalin in the conditioned media, nor did we find the cells to express DA β-hydroxylase, which catabolizes DA to noradrenalin. These data suggest that at least some of the induced cells were dopaminergic directed. While we could not detect DA following the differentiation protocol used, we recently reported that supplementing the same induction protocol with neurotrophic factors such as BDNF and GDNF results in DA secretion [12]. Thus we refer to some of the induced cells in this study as dopaminergic precursors.

Previous work has already demonstrated the beneficial effect of MSC to the damaged central nervous system [43–45]. The main goal of this study was to prove the added value of transplanting neural-induced cells in the rat PD model over transplanting naive hMSC. The significant improvement in rotational behavior, which was observed only in the group transplanted with the induced cells, indeed confirmed our hypothesis that differentiation prior

to transplantation facilitates the therapeutic potential of hMSC.

Identifying the exact mechanism by which the beneficial behavioral effect was accomplished is a tricky task. However, immunohistochemical analysis revealed that the neural-induced cells were indeed present within the lesioned striatum more than 130 days following transplantation, showing integration into the brain parenchyma, survival and even migration toward the ipsilateral nigra. Recent reports describing the therapeutic benefit of MSC attribute their effect to various mechanisms. One possibility is functional cell replacement [46], another is a restorative effect of the transplanted cells on the remaining neurons within the host brain, perhaps through secretion of neurotrophic factors [47]. A third possibility is immunomodulation of the host response to the lesion [48] and finally, some reports suggest that the transplanted MSC enhance endogenous neurogenesis [49]. In our work, it would be fair to assume that at least part of the therapeutic effect could be attributed to functional cell replacement. Considering the dopaminergic precursor phenotype exhibited *in vitro* and after finding that some of the transplanted cells expressed TH within the damaged striatum, we postulate that dopaminergic cell replacement indeed occurred to some extent. Moreover, we employed the more severe 6-OHDA model, injecting the toxin to the medial forebrain bundle, in which an almost complete denervation of the nigrostriatal pathway is achieved. A restorative effect in this model is less likely as very few of the endogenous dopaminergic cells remain. That is in contrast to the 6-OHDA injection in the striatum, which is commonly used to evaluate the neurotrophic effect.

We cannot rule out any other possible mechanism suggested above. Still, the fact that naive MSC proved to be less efficient in ameliorating the rotational behavior clearly indicates that the induced cells performed better in at least one of the proposed mechanisms, replacement, restoration, immunomodulation or induction of endogenous neurogenesis. Future studies would have to address the challenge of elucidating the cells' mechanism of action.

To conclude, our results demonstrate that neural induction enhances the therapeutic potential of hMSC. The induction protocol resulted in the generation of cells exhibiting several traits of dopaminergic precursors, which upon transplantation induced behavioral improvement in hemi-Parkinsonian rats. We hope that our result will serve as a first step toward hMSC-based cell therapy for PD.

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