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Intranigral Transplantation of Epigenetically Induced BDNF-Secreting Human Mesenchymal Stem Cells: Implications for Cell-Based Therapies in Parkinson's Disease

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It is thought that the ability of human mesenchymal stem cells (hMSC) to deliver neurotrophic factors might be potentially useful for the treatment of neurodegenerative disorders. The aim of the present study was to characterize signals and/or molecules that regulate brain-derived neurotrophic factor (BDNF) protein expression/delivery in hMSC cultures and evaluate the effect of epigenetically generated BDNF-secreting hMSC on the intact and lesioned substantia nigra (SN). We tested 4 different culture media and found that the presence of fetal bovine serum (FBS) decreased the expression of BDNF, whereas exogenous addition of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to serum-free medium was required to induce BDNF release (125 ± 12 pg/day/10^6 cells). These cells were called hM(N)SC. Although the induction medium inhibited the expression of alpha smooth muscle actin (ASMA), an hMSC marker, and increased the nestin-positive subpopulation of hMSC cultures, the ability to express BDNF was restricted to the nestin-negative subpopulation. One week after transplantation into the SN, the human cells integrated into the surrounding tissue, and some showed a dopaminergic phenotype. We also observed the activation of Trk receptors for neurotrophic factors around the implant site, including the BDNF receptor TrkB. When we transplanted these cells into the unilateral lesioned SN induced by striatal injection of 6-hydroxydopamine (6-OHDA), a significant hypertrophy of nigral tyrosine hydroxylase $(TH)^+$ cells, an increase of striatal TH-staining and stabilization of amphetamine-induced motor symptoms were observed. Therefore, hMSC cultures exposed to the described induction medium might be highly useful as a vehicle for neurotrophic delivery to the brain and specifically are strong candidates for future therapeutic application in Parkinson's disease.

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INTRODUCTION

Bone marrow (BM) contains mesenchymal stem cells (MSCs), also termed multipotent stromal cells, with the ability to differentiate in vitro into mesenchymal and nonmesenchymal lineages, including neuronal and glial lineages [1-8]. MSCs are potentially useful for transplantation in several neurodegenerative diseases, but the mechanisms responsible for a beneficial outcome are not fully understood [9]. A possible mechanism involved in the neuroprotective effect of MSCs is neural transdifferentiation. However, it is currently thought that the therapeutic benefits of MSCs depend on their ability to produce soluble factors that can promote neuronal cell survival and neuritogenesis [10,11], stimulation of axonal growth [12,13], functional recovery [14,15], and modulation of microglial activation [16]. At present, the positive behavioral effects observed after MSC transplantation in the striatum, which is the usual target implantation region of parkinsonian animal models, cannot be explained by the low/insufficient number of dopaminergic donor neurons derived from the grafted cells [17-19], but it may depend on immunomodulation and/or growth factor delivery [9,11,17,20].

Parkinson's disease (PD) is characterized by the degeneration of nigrostriatal dopaminergic neurons

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of the lateral substantia nigra pars compacta (SNpc) [21] and is associated with reduced levels of neurotrophins such as brain-derived neurotrophic factor (BDNF) [22-24]. Dopaminergic neurons of the SNpc depend on BDNF [25]. BDNF is produced mainly in nigral neurons [26], which also express its receptor TrkB [27]. Infusion of BDNF into the SNpc region promotes survival of axotomized nigrostriatal dopaminergic neurons [28]. Additionally, intranigral antisense oligonucleotide infusion indicated that loss of BDNF expression leads to dopaminergic neuronal death [29]. Herein, we suggest that BDNF supplemented by cells implanted in a nigral location could be better suited for a neurotrophin-based therapy for PD than a striatal placement [30].

Some previous results indicate that hMSC in culture express BDNF using standard and induction conditions [10,11,20,31-34], but other reports observed that significant BDNF release is obtained only after BDNF gene transfer into hMSC [13,35-37]. To date, the variability of results published with regard to the levels and types of neurotrophins produced by hMSC could be explained by the age of the human donors or the method used to isolated hMSC from BM [38]. In addition, the presence of fetal bovine serum (FBS) in the medium is the most variable factor contributing to heterogeneous results because of its composition and endogenous levels of growth factors and cytokines, which can vary based on the batch and quantity used [39]. These factors could mildly modify the biology of the hMSC (eg, the proteins they express and/or secrete). We previously observed BDNF in conditioned media obtained from hMSC cultures isolated by plastic adhesion after exposure to a defined serum-free medium [34]. In the present study, we evaluated the effects of FBS supplemented with both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on gene expression, cell proliferation, and the ability to express and/or release BDNF. We also evaluated the in vivo effect of BDNF-secreting hMSC, called hM(N)SC, in the intact and lesioned rat SNpc. Induced human cells integrated perfectly into the rat SN, some of which differentiated into a dopaminergic phenotype and activated Trk receptors at the implant site. Our data suggest that epigenetically generated hM(N)SC exert neurotrophic effect on the impaired dopaminergic nigrostriatal system.

MATERIALS AND METHODS

Isolation and Expansion of hMSC Cultures

hMSC cultures were isolated by plastic adhesion and characterized after 1 passage as described previously [34]. After informed consent, BM aspirates were collected from 5 normal young individuals undergoing BM harvest for allogeneic transplantation, as part of a protocol approved by the Ethical Committee of the Hematology Department of Clinica Alemana (Santiago, Chile). Total cells were seeded at a density of 1×10^6 nucleated cells/cm^2 in α-minimal essential medium supplemented with 10% FBS and 0.8 mg/L gentamycin (MSC-medium). After 24 hours, nonadherent cells were removed by replacing the culture medium. When the foci of fibroblast-like cells were confluent, the cells were detached with 0.25% (p/v) trypsin and 2.65 mM EDTA and subcultured at 7×10^3 cells/cm^2 for further expansion. Isolated cells were characterized by real-time RT-PCR for CD34, CD105, and alpha smooth muscle actin (ASMA), and their capacity to differentiate in vitro into adipocytes, chondrocytes, and osteocytes was evaluated as described previously [34].

Culture Media Composition

To evaluate the effect of FBS and specific growth factors (EGF and bFGF), we cultured cells in MSC-medium, MSC + GF-medium (MSC-medium with human EGF plus human bFGF, both at 10 ng/mL [R&D Systems, Minneapolis, MN]), NSC-medium (DMEM/F12 [1:1] [Gibco, Grand Island, NY], 1% bovine serum albumin [BSA, Merck, Rahway, NJ], 6 g/L D[+]-glucose [Merck], 0.8 mg/L gentamicin supplemented with N2 and B27 supplements [Gibco]) and NSC + GF-medium (NSC-medium supplemented with EGF and bFGF, 10 ng/ml of each) for 3 and 8 days.

Cellular Viability and Proliferation

To examine cell viability and proliferation, we used a colorimetric assay using WST-1 reagent (Roche, Germany), a tetrazolium salt that is converted into formazan dye by mitochondrial dehydrogenases. Cells were seeded at a density of 4×10^3 cells/cm^2, and 2 to 4 hours later, WST-1 was diluted 1/10 (v/v) in the respective serum-free media and incubated for 4 hours at 37°C. The net absorbance at 450 nm was normalized with respect to day 0.

RT-PCR Analysis

Total RNA was isolated from cells using TRIZOL® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 1 μg of total RNA was used for reverse transcription (RT). PCR was performed in a capillary containing 100 ng of cDNA, PCR LightCycler-DNA Master SYBR Green reaction mix (Roche, Indianapolis, IN), 3-4 mM $MgCl_2$, and 0.5 mM of each specific primer, using a LightCycler® thermocycler (Roche). For primer sequences and control human cell lines, see the description from a previous article [34]. Negative controls without reverse transcriptase were performed. Negative PCR results were validated by amplification of the housekeeping gene GAPDH. PCR sensitivity for each neural gene was calculated previously [34].

Conditioned Medium

hMSC were seeded in MSC-medium at a density of 7×10^3 cells/cm^2. Cells were allowed to attach to the dish for 2 to 4 hours, and then the MSC-medium was replaced with the 4 corresponding types of media. After 48 hours, the media were collected and filtered using 0.2-μm pore filters (Orange Scientific, Belgium).

BDNF Quantification

Samples were assessed for BDNF concentrations using the BDNF Emax ImmunoAssay System Kit (Promega, Madison, WI) according to the manufacturer's protocol. A standard curve of recombinant human BDNF ranging from 7.8 to 500 pg/mL was determined for each titer plate. The absorbance values were expressed as pg of BDNF produced by a million cells in 24 hours. BDNF standards and experimental samples were performed in triplicate, and the BDNF concentration was calculated from absorbance values at 450 nm, minus the absorbance of the corresponding cell-free culture media.

Immunodetection

We used different antibodies to detect cell-specific proteins. Nestin was detected in cells by both immunofluorescence and flow cytometry with a mouse monoclonal antibody (mAb; 1:100, R&D System); BDNF protein in cell cultures was detected with a rabbit polyclonal antibody (pAb) (1:1000. Santa Cruz Biotechnology, Santa Cruz, CA); grafted hM(N)SC in brain sections was detected using a mouse mAb against hRNP (1:100, Chemicon, Temecula, CA); dopaminergic neurons were detected with an anti-TH mouse mAb antibody (1:1000, Sigma, St. Louis, MO) for immunohistochemistry or anti-TH rabbit pAb antibody (1:5000, Chemicon) for immunofluorescence; and high-affinity BDNF receptor and phospho-Trk receptors were detected in brain sections using rabbit pAb TrkB (1:800, BD Transduction Laboratories, San Jose, CA) and mouse mAb phospho-TrkA (Tyr490) (1:400, Cell Signaling, Danvers, MA), respectively. The rabbit antibody against phospho-Tyr-816 of TrkB was kindly provided by Moses Chao and used at a 1:100 dilution. For immunohistochemistry, primary antibodies were detected with biotinylated antimouse secondary antibody (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) using a nickel-intensified reaction according to the published protocol [40]. Immunofluorescence for nestin was done using a goat antimouse PE-conjugated secondary antibody (1:100, BD Biosciences, Mountain View, CA). Double immunostaining for nestin/BDNF, hRNP/TH and TrkB/pTrkA was carried out with specie-specific secondary antibodies coupled to Alexa Fluor®488 and Alexa Fluor®555 (1:200, Invitrogen, Eugene, OR).

Flow Cytometry

A total of 1×10^6 cells were fixed in 70% ethanol. Cell fractions of 2×10^5 cells were resuspended in PBS containing 2% FBS and first incubated with primary antibody for 60 minutes at 4°C, washed twice, and then incubated with PE-conjugated secondary antibody for 60 minutes at 4°C (for antibody specifications see the Immunodetection section). Cell fluorescence was evaluated by flow cytometry using FACScan (BD Biosciences), and the data were analyzed using CellQuest software (BD).

Cell Nigral Transplantation and 6-Hydroxydopamine Injection

Surgical procedures were performed under the supervision of the Animal Care Committee at the Universidad del Desarrollo, Chile. Care of animals and protocols were in accordance with institutional guidelines. Male Sprague Dawley rats (Universidad de Chile, Santiago, Chile), weighing 220-280 g, were anesthetized with a mix of ketamine (50 mg/kg) and xilacine (10 mg/kg). Using a stereotactic frame and a 10-μL Hamilton syringe we used the following coordinates from rat brain atlas [41]. For nigral transplantation we injected 4 μL of phosphate buffer solution (pH 7.4) containing 5% of heterologous rat serum (vehicle) or hM(N)SC (5×10^4 cells/μL) at 2 depths ventral to the SNpc (from bregma, AP, anteroposterior; ML, mediolateral; V, vertical from dura). AP −5.0 mm, ML −1.6 mm, V −8.4, and −8.2 mm, tooth-bar = −3.3. For the experiment in lesioned SN, 1 week before cell transplantation were injected with 6 μL of 6-OHDA (4 μg/μL) in 0.02% (+)-sodium L-ascorbate (Sigma) of saline solution distributed in 3 sites into the right dorsal Str: AP −1.0 mm, ML −3.5 mm, V −5.0, −4.5, and −4.0 mm, tooth-bar = 0. Animals were immunosuppressed by daily injections of dexamethasone (initial dose of 0.5 mg/kg subcutaneously), starting 1 day before transplantation, until the end of the experiment (1 week for intact animals and 3 weeks for lesioned animals).

Histology

Brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed as described previously [40]. Serial coronal sections of 30 μm or 40 μm were collected for immunofluorescence or immunochemistry assays, respectively. Brain slices were visualized under a light microscope (AxiosKop, Zeiss, Germany) or a fluorescence confocal microscope (Olympus Fluoview FW1000).

Estimates of Sprouting and Motor Function Assessment

For this aim, we use a relative index of extent of fiber innervation based on the validity of densitometric

measures of striatal TH as previously described [42]. The TH-staining area was measured based on segmentation on the basis of gray level of the stained region and the background pixels from brain section images using NIH Scion Image software. To selectively detect TH immunoreactivity the thresholding lower and upper gray values were set at 152 and 252, respectively.The sprouting index was calculated by dividing the immunopositive area in the Str by the number of TH^+ cells in the SNpc. Rotational motor response was analyzed in both lesioned groups (lesioned-sham and lesioned-grafted) with methamphetamine hydrochloride (5 mg/kg/dose intraperitoneally (i.p.), Cidrin, Abbott Laboratories, Chile) at 1 week after the 6-OHDA intrastriatal injection and 3 weeks after vehicle or hM(N)SC intranigral injection. The rotational behaviors (360 degree turns) were assessed for 30 minutes with a video camera and the analyses were based on the net scores (ipsilateral minus contralateral rotations).

Quantifications

For SN analysis, cell counts and soma area were obtained from a total of 5-10 sections analyzed per animal, restricted to interaural 4.20 mm/bregma −4.80 mm and interaural 2.96 mm/bregma −6.04 mm. Data from soma area was restricted to cells >124 μm^2. Statistical significance for comparing hemispheres was determined using the nonparametric Mann-Whitney test. For Str analysis, striatal area were analyzed from 5 to 10 sections per animal restricted to interaural 10.00 mm/bregma 1.00 mm and interaural 8.20 mm/bregma −0.80 mm [43]. A nonparametric Mann-Whitney test was used for AIR test analysis. Statistical analysis were done using the Software SPSS15 (SPS, Chicago, Inc.), and all data were processed using the standard error mean (SEM).

RESULTS

Effect of Different Culture Media on the Biologic Properties of hMSC Cultures

We tested 4 different media to obtain a protocol that should allow the generation of BDNF-secreting cells from hMSC cultures: serum-containing medium that has been used to isolate hMSC from BM [34,44] (MSC-medium), serum-free medium that has been used to isolate neural stem cells from the olfactory bulb [34,45] (NSC-medium), and their corresponding media supplemented with exogenous EGF and bFGF (Growth Factors, GF; MSC + GF-medium and NSC + GF-medium).

Cells grown in the serum-containing media (MSC and MSC + GF) had similar fibroblast-like morphologies, whereas cells in the serum-free media (NSC and NSC + GF) were mostly enlarged and flattened

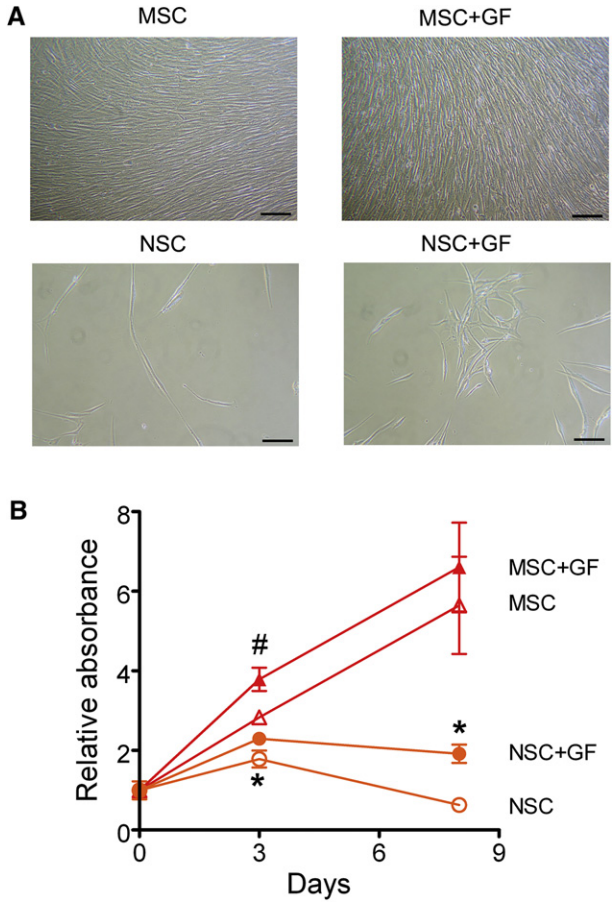

Figure 1. Characterization of hMSC cultures in four different culture media. (A) Representative images taken to evaluate cell morphology and the number of cells after 8 days in culture. Scale bars: 100 μm. (B) Proliferation curve based on absorbance measurements (450 nm) from the WST-1 test. All four groups were different from each other at day 3 (#, $P < .05$). The serum-containing media (MSC and MSC + GF) were able to expand cells from hMSC cultures until at least day 8. The serum-free media (NSC and NSC + GF) arrested cell proliferation after the third day, being more pronounced in the absence of exogenous growth factors (NSC-medium) at days 3 and 8 (*$P < .05$). The data are the mean ± SEM of absorbance values obtained from four independent cultures measured.

by day 8 (Figure 1A). Doubling times at day 3 ranged from 24 to 72 hours in the following sequence: MSC + GF-medium < MSC-medium < NSC + GF-medium < NSC-medium (Figure 1B). From 3 to 8 days, both MSC-type serum-containing media yielded vigorously proliferating cultures compared to NSC-type serum-free media. As shown, hMSC proliferation in serum-free media decreased quickly between the third and the eighth day, regardless of the presence of GF. However, cell viability only decreased at day 8 when cultures were grown in the absence of both FBS and exogenous GF (NSC-medium: 3 days, 1.78 ± 0.20; 8 days, 0.63 ± 0.10; $P = .05$; Figure 1B). We observed a multineural gene expression pattern in undifferentiated culture conditions (MSC-medium; real-time RT-PCR data, Table 1), as described previously [34]. The addition of EGF and bFGF to the

Table 1. Effect of Media on the Gene Expression Profile* of hMSC Cultures† at Days 3 and 8

Medium	Day	ASMA	Nestin	NF-M	GFAP	GalC	GAPDH
MSC	3	+	+	+	$+^{3}$	$+^{3}$	+
	8	+	+	+	+	+	+
MSC + GF	3	+	+	+	+	$+^{3}$	+
	8	$+^{3}$	$+^{3}$	$+^{3}$	$+^{3}$	$+^{3}$	+
NSC	3	$+^{2}$	$+^{2}$	+	$+^{2}$	$+^{3}$	+
	8	–	–	+	$+^{1}$	$+^{2}$	+
NSC + GF	3	–	$+^{3}$	+	$+^{2}$	$+^{2}$	+
	8	–	$+^{2}$	+	$+^{1}$	$+^{3}$	+

ASMA indicates alpha-smooth muscle actin; NF-M, medium chain neurofilament; GFAP, glial fibrillary acidic protein; GalC, galactocerebroside; GAPDH, glyceraldehyde 3-phosphate dehydrogenase as a housekeeping gene; MSC, mesenchymal stem cells.

+, detected; –, nondetected; numbers in superscript indicates the number of positive samples; absence of superscript numbers indicate 4 of 4 positive or negative results.

*Mesenchymal marker (ASMA) and neural markers (Nestin, NF-M, GFAP, GalC).

†From 4 different donors analyzed by RT-PCR in four different types of media.

medium (MSC + GF) increased the cell proliferation rate but did not change their fibroblast morphology and gene expression pattern. In contrast, serum-free media affected the gene expression pattern and morphology of hMSC (Table 1 and Figure 1A). Although neuronal and glial genes such as NF-M, GFAP, and GalC were expressed in all conditions (Table 1), the expression of the mesenchymal marker ASMA was undetectable by RT-PCR, and the nestin gene was only expressed in cells cultured in NSC + GF-medium. We then quantified nestin^{+} cells by flow cytometry from cultures exposed to MSC-medium (hMSC) or NSC + GF-medium (hM(N)SC). The nestin^{+} cell subpopulation remained stable throughout the time of the experiment (from day 3 until day 8) only when cultured in media supplemented with growth factors (Figure 2A). Moreover, the NSC + GF-medium induced nearly a 2-fold increase in the percentage of nestin^{+} cells after 3 days (flow cytometry data: MSC-medium, 26.9 ± 6.1%, n = 4; NSC + GF-medium, 50.5% ± 3.5%, n = 3; $P = .0286$). In addition, we evaluated the effect of the 4 types of media on the synthesis and secretion of BDNF. In the supernatant of hMSC cultures exposed to NSC + GF-medium, we detected a BDNF release rate of 125 ± 12 pg/24 hour/10^{6} cells (Figure 2B). BDNF-expressing cells had a neuronal-like shape and did not express nestin after 3 days, in contrast to the fibroblast-like morphology of nestin^{+} cells

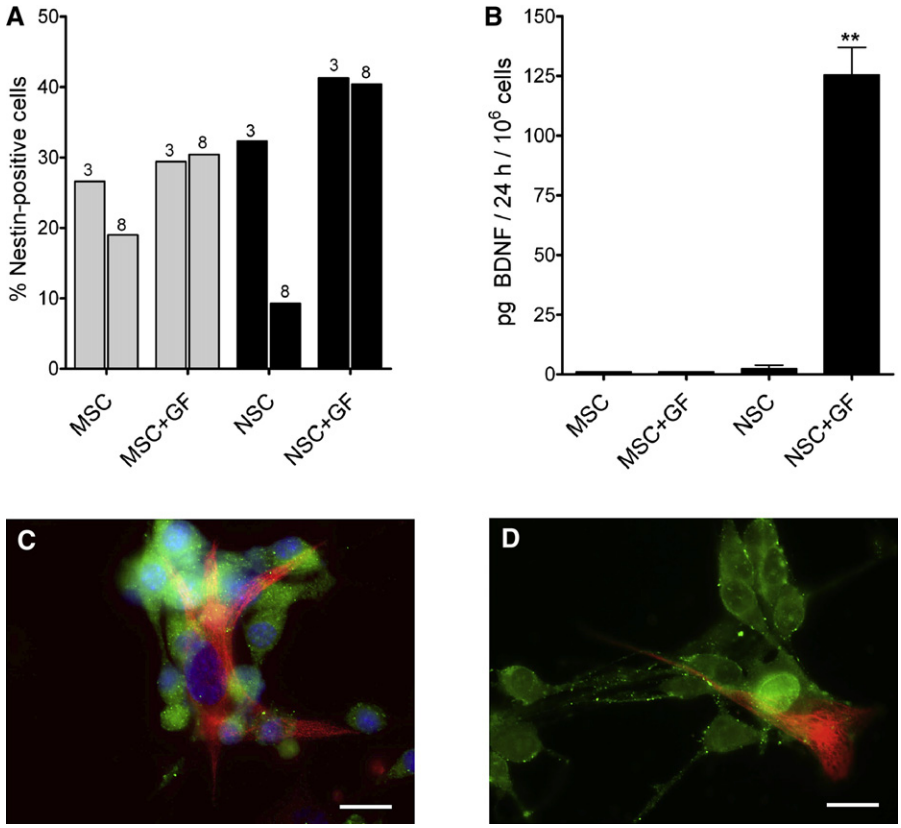

Figure 2. The application of NSC + GF-medium induces BDNF secretion in hMSC cultures and increases the percentage of nestin-positive cells. (A) Flow cytometry data from a representative hMSC culture indicate that the percentage of nestin^{+} cells was maintained in media supplemented with growth factors (MSC + GF and NSC + GF) between the third (3) and eighth (8) day. (B) Effect of different media on the generation of BDNF-releasing hMSC. BDNF levels were measured by ELISA using supernatant obtained from conditioned medium (**$P < .01$, n = 3, Student's *t*-test). (C-D) Immunofluorescence images show immunolabeling with anti-nestin (red) and anti-BDNF (green, C) or anti-phospho-TrkB (green, D) of hMSC cultures after 3 days in culture with MSC-medium (hMSC) or NSC + GF-medium [hM(N)SC]. There were two different subpopulations of cells, as suggested by the absence of colocalization of both proteins. Cell nuclei were stained with DAPI (C, blue). Scale bar: 20 μm.

(Figure 2C). In addition, the TrkB receptor was phosphorylated in nestin-negative cells (Figure 2D). Altogether, these data indicated that the application of NSC + GF-medium for 3 days constitutes an appropriate neural induction protocol to generate hMSC cultures with an increased nestin$^+$-subpopulation and BDNF-secreting cells with activated TrkB receptors. We therefore named the hMSC cultured with this medium hM(N)SC.

Effect of Grafted hM(N)SC into the Intact SN

Based on the above data, we generated hM(N)SC by applying the neural induction protocol to hMSC cultures to transplant them into the SN for in vivo characterization (Figure 3A). Before examining whether hM(N)SC might release BDNF in vivo in the SN and exert a neurotrophic effect on dopaminergic neurons, we evaluated basic transplantation parameters like cell survival and engraftment. We detected surviving human cells throughout the ventral right SNpc after immunostaining for human ribonucleoprotein [40] (hRNP, Figure 3B). The hM(N)SC grafts did not disrupt the normal cytoarchitecture of the brain; there was no sign of cell overgrowth or tumor formation at the level of SN in any of the experimental animals (Figure 3C), and a discrete lateral migration, 0.5-1 mm away from the injection site, was observed (Figure 3B). The number of TH$^+$ cells did not change for any grafted animals (Figure 3D). We observed immunolabeling of phosphorylated-Trk receptors around the implant site that colocalized partially with TrkB (right, Figure 4A). Additionally, we observed a trend of an increased area of TH$^+$ cells, which was significant for the intermedia SNpc region of 1 animal (Figure 4B). We also found occasional donor human cells expressing TH at the implant site (Figure 4C). In summary, we observed that hM(N)SC grafted well into the SN after 1 week and had a neurotrophic effect on the intact SNpc.

Effect of Grafted hM(N)SC into the Lesioned SN

At this point, we asked whether intranigral transplantation of hM(N)SC could exert an effective neurotrophic support on nigrostriatal dopaminergic pathway. We injected either vehicle (n = 6) or cells (2×10^5 cells, n = 7) into the partially lesioned SNpc at 1 week postinjection with 6-OHDA and evaluated the effects after 3 weeks, according to the experimental design (Figure S1). Similar to the observations shown earlier for transplantation into the intact SN, we detected human cells (Figure 5B) and immunolabeling for TrkB and phosphorylated-Trk receptors after transplantation into the lesioned SN (Figure 5C). However, we did not find human cells expressing TH (data not shown). Although both hemispheres showed a similar number of nigral TH$^+$ cells per section (intact side: hM(N)SC, 252 ± 53, vehicle, 225 ± 33; lesioned side: hM(N)SC, 101 ± 172, vehicle, 105 ± 31), the sprouting

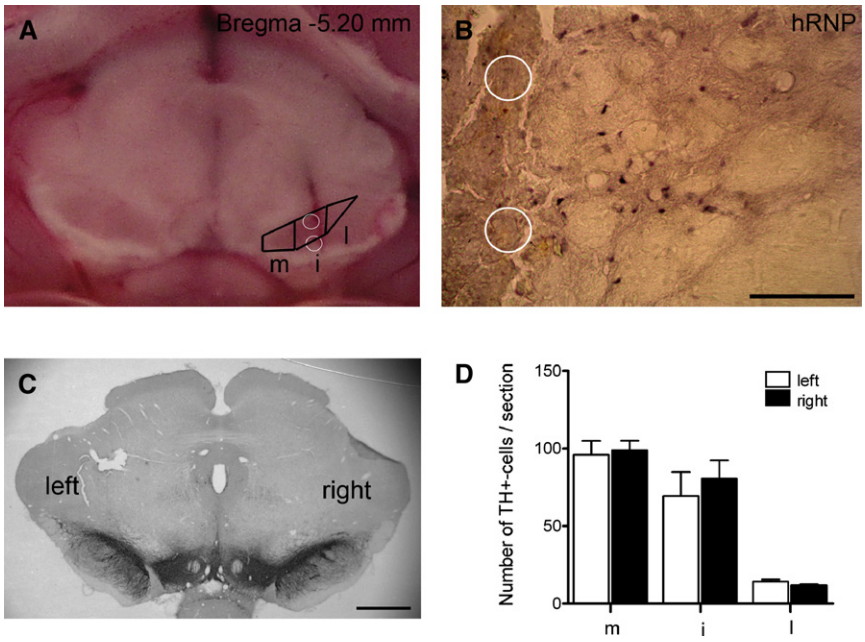

Figure 3. Nondisruptive integration of hM(N)SC cell transplants without deleterious effect on SNpc dopaminergic neurons. (A) The image shows a coronal view of fresh brain following injection at the level of SN (see coordinates in Methods) to examine the implant location. Circles indicate the implantation sites at the level of the intermedia (i) region. Within the boxed areas: m, i, and l, indicate medial, intermedia, and lateral region of the SNpc, respectively. (B) Immunolabeling with anti-hRNP (black) showed the survival and moderate migration of human cells in the rat SN. Scale bar: 200 μm. (C) Representative TH-immunostaining of a brain section at the level of the cell transplant and (D) quantification of total TH$^+$ cells in the nongrafted (left) and grafted (right) hemispheres of the SNpc 1 week postgrafting. Scale bar: 1 mm.

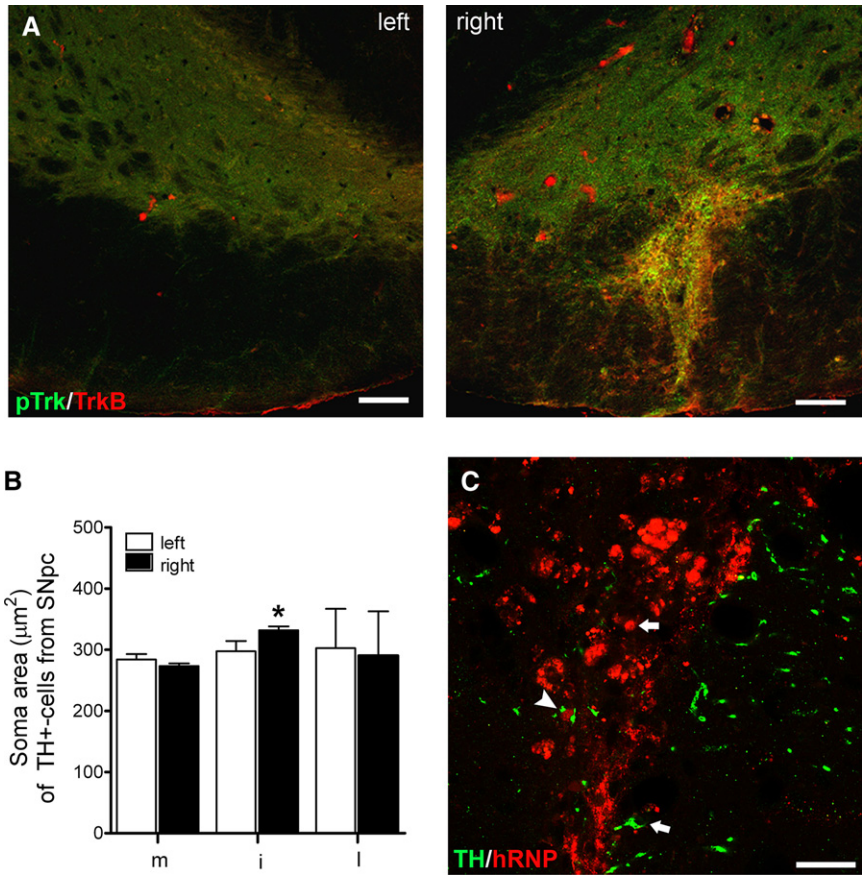

Figure 4. In vivo effect of BDNF-secreting hMSCs into the intact SNpc 1 week postgrafting. (A) Images obtained by confocal microscopy after double immunolabeling with anti-phospho-Trk (green) and anti-TrkB (red) antibodies in the left and right hemisphere of a grafted rat brain. Colocalization of fluorescence (yellow) indicates highly probable activation of TrkB receptors on the right (transplanted) side. Scale bar: 500 μm. (B) Soma area was calculated for a total of 100-400 TH^+ cells for each SNpc region (m, medial; i, intermedia; l, lateral; $^*P < .05$). (C) Representative composition from serial confocal images at the level of the implant site shows some grafted cells expressing the dopaminergic maker TH. The top arrow indicates a human cell (red, nuclear label), whereas the inferior arrow indicates an endogenous dopaminergic cell (green, cytoplasmic label). The arrowhead indicates a human TH-expressing cell (red nucleus, green cytoplasm). Scale bar: 100 μm.

index, calculated to estimate fibber innervations of nigrostriatal neurons, was increased in the right Str of lesioned-grafted rats after 3 weeks compared to the sham group (Figure 5D). Additionally, we measured the soma area of nigral TH^+ cells from SNpc and we found greater values in the right side of lesioned-grafted rats (Figure 5E). In terms of behavior, the number of amphetamine-induced rotations increased significantly after 3 weeks only in the lesioned-sham group with respect to pregrafting data (126.7% of average increase), but not in the lesioned-grafted rats (Figure 5F), suggesting that neurodegeneration progressed more in nongrafted rats. Altogether, these data suggested that hM(N)SC could be exerting a specific neurotrophic effect on the dopaminergic neurons of the lesioned SNpc.

DISCUSSION

The first relevant finding of the present study is that hMSC are effectively induced to express and release BDNF after being cultured in the serum-free medium, NSC + GF, and that this effect seems to be restricted to the nestin-negative subpopulation. The signals involved in BDNF induction include the absence of FBS and the presence of EGF and bFGF. Second, it is the first time that hMSC-derived cells implanted into the SN survived and integrated well into the host to form a chimeric tissue. Human cells remained close to the implant site and did not disrupt the SNpc architecture. We also observed signs of neuronal transdifferentiation in some human cells implanted into the intact, but not lesioned, SN that expressed the dopaminergic marker TH, although the total number of TH^+ cells remained unchanged. Additionally, we observed hypertrophy of nigral cells and graft-dependent activation of TrkB and other Trk receptors at the implant site, suggesting that grafted hM(N)SC could deliver bioactive BDNF into both the intact and lesioned SN. The third important finding is that intranigral cell grafts did not alter the number of dopaminergic cells in the SNpc but exerted a neurotrophic support

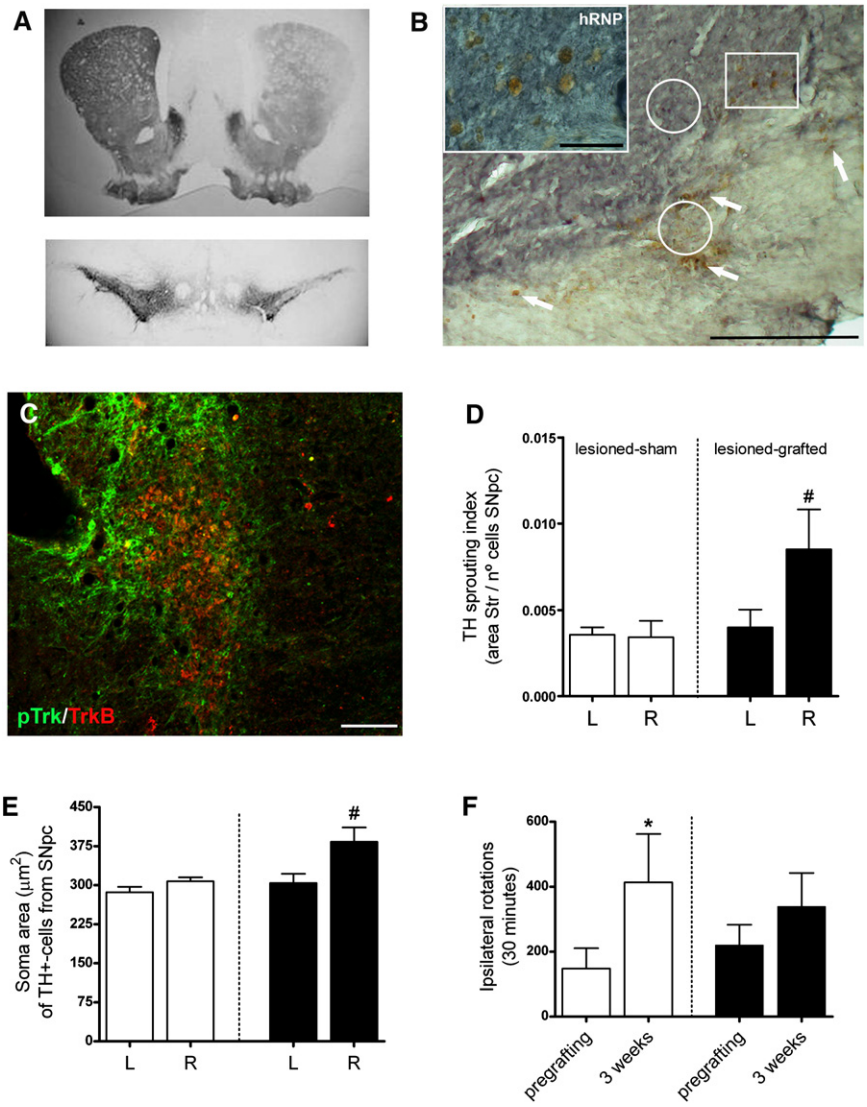

Figure 5. Effect of hM(N)SC on the lesioned nigrostriatal pathway. (A) Representative sections show TH staining of Str and SNpc after 4 weeks of 6-OHDA striatal injection and 3 weeks after intranigral cell transplantation into the right hemisphere. (B) Specific human anti-hRNP antibody revealed grafted cells at different places around the implant site (white arrows) in the right SNpc of a lesioned-grafted rat. The circles indicate the 2 injection sites. Scale bar, 250 μm. High magnification of boxed area shows cells (brown) perfectly integrated into the brain tissue 3 weeks postimplantation. Scale bar, 100 μm. (C) Representative confocal image obtained from the right SNpc of a lesioned-grafted rat shows TrkB (red), phosphorylated Trk receptors (green), or phosphorylated TrkB, resulting from merging of red and green fluorescence (yellow) at the implant site. Scale bar, 250 μm. (D) Graphic represents the sprouting index calculated from TH staining data. For the lesioned-grafted group, a significant difference were found between the intact (L, left) and lesioned (R, right) side and with respect to the lesioned-sham group ($^{\#}P < .05$). (E) Hypertrophy of TH^{+} cells was induced in the right SNpc of the grafted rats (black bars) with respect to the sham rats (white bars) ($^{\#}P < .05$). (F) The number of amphetamine-induced rotations was compared with the baseline measurement of each rat (pregrafting condition: 1 week postlesion). Three weeks postgrafting the number of AIRs increased ($^{*}P < .05$) for the lesioned-sham group (white bars) but not for the lesioned-grafted group (black bars).

on the ongoing degeneration of the nigrostriatal dopaminergic system as suggested by the hypertrophy of nigral somas and the increased innervated area in the striatum.

The ability of undifferentiated hMSC to express neurotrophic factors in FBS-containing medium is controversial. Here, we were able to induce BDNF release by hMSC cultures when cultured in NSC + GF, at levels 3- to 10-fold higher than in other studies using serum-containing medium [10,11]. In agreement with this, we detected nestin-negative cells with low BDNF levels when we used medium containing 10% FBS (Figure S2). This was undetectable by our ELISA assay (with a sensitivity limit of 7.8 pg/mL).

The mechanisms that modulate BDNF release in hMSC are not fully understood. In addition, hMSC cultures are composed of heterogeneous populations of mesenchymal progenitors with different commitments, as described before in clonal studies [34,46]. Our hMSC cultures grown in serum-free media (NSC or NSC + GF) had downregulated levels of early neural and mesenchymal markers (nestin and/or ASMA,

respectively) and expressed glial and neuronal markers (Table 1). Moreover, the subpopulation of BDNF$^+$ cells were grouped, had a neuronal-like morphology, and were nestin-negative. This could indicate a more mature neural state of cells, as was found in adult human olfactory neural progenitors after the addition of N2 and B27 supplements to a serum-free medium [47]. Moreover, BDNF is upregulated under adverse conditions such as serum deprivation in neuronal cultures [48]. Therefore, we postulate that in the nestin-negative subpopulation, reflecting cells more committed toward a neuronal fate, serum deprivation in the presence of EGF and bFGF leads to phosphorylation of the transcription factor CREB and subsequently to BDNF transcription, synthesis, and release as reported for cells of neural origin [48-50].

Nestin is a marker of an undifferentiated neural state and is expressed in human neural progenitor cells (hNPC) [51]. It is now accepted that immature hMSC from adult bone marrow usually express nestin as well [34,52-54]. Therefore, EGF and bFGF, which were present in our induction medium, could be acting as survival factors in uncommitted nestin$^+$ cells, as it has been shown in hNPC cultures [51]. However, BDNF secreted by nestin-negative cells would have the opposite effect on nestin expression [55], as suggested previously in a clonal study [34]. This could be mediated by TrkB activation that occurs exclusively in nestin-negative cells as we observed after applying our neural induction protocol, but not a standard culture medium (see Figure S2). Moreover, BDNF added to defined differentiation medium results in dopaminergic differentiation of hMSC [56]. Therefore, BDNF secreted by hMSC-derived nestin-negative cells could induce neuronal self-differentiation.

BDNF could synergize with other neurotrophins that act through the Trk receptor family [57,58]. NGF is the ligand for TrkA, whereas BDNF and NT-4/5 bind to TrkB, and NT-3 binds to TrkC. BDNF and NT-3, but not NGF, have roles in the survival and function of dopaminergic neurons [59]. BDNF also can induce hypertrophy of dopaminergic cells [60]. The hypertrophic effect of grafted hM(N)SC on dopaminergic neurons together with the detection of phospho-Trk receptors at the implant site both in the intact and lesioned SNpc sugests a BDNF-mediated effect. Additionally, we coimmunodetected TrkB in 97%-100% nigral TH$^+$ cells. However, we cannot exclude the possibility that other neurotrophic factors, such as GDNF, were released by our cells and affected nigral dopaminergic cells. Indeed, another study described hypertrophy or sprouting of nigral dopaminergic cells when implanting GDNF-secreting fibroblasts in the dorsal SNpc [61]. Furthermore, we found that striatal TH-staining area was higher in lesioned-grafted with respect to lesioned-sham animals at 4 weeks postlesion. This result, together with Trk activation at the implantation site and hypertrophy of nigral TH$^+$ cells, suggest that cells release neurotrophins (eg, BDNF) in vivo that exert a positive action on the degenerating nigrostriatal dopaminergic pathway. Although it has been published recently that intrastriatal administration of 6-OHDA produced an early and transient augmentation of BDNF expression at 1 week [62], we did not find TrkB activation in non-grafted SNpc at 4 weeks postlesion (data not shown). However, the enhancement of striatal sprouting and soma area in the lesioned side of grafted animals suggests that BDNF, among other neurotrophic factors, could be directly promote the survival, hypertrophy, and sprouting of dopaminergic neurons or even facilitate the release of DA from host DA neurons as previously reported [63]. As the injection of 6-OHDA in the Str produces a progressive model of PD that needs about 3 to 4 weeks to complete the process [64], our histologic data suggest that the hM(N)SC might protect or rescue impaired dopaminergic neurons in the SNpc, thereby improving motor deficits.

Ourednik and collaborators [65] showed that intranigral grafts of the mouse NSC line C17.2 can promote sprouting of spared nigrostriatal dopaminergic efferents and induce functional recovery of the dopaminergic neurons after transplantation into the partially lesioned SN, an effect that might probably mediated by GDNF. However, they did not show GDNF receptor activation in the host tissue. Previously, we described that C17.2 cells could release 55 pg/24 hour/10^6 cells of BDNF in the absence of any genetic modification [66]. Therefore, the functional effects in PD models observed using this cell line [65] could also be mediated by BDNF/TrkB signaling.

With respect to a possible therapeutic effect, the stabilization of rotations observed in the grafted rats between pregrafting and 3 weeks postgrafting is in accordance with higher TH immunostaining in Str of grafted versus sham animals. Thus, we postulate that grafted cells were able to halt/slow down the nigrostriatal dopaminergic degeneration. As we did not find TH expression in the grafted human cells detected in the lesioned SNpc, the effect of grafted hM(N)SC in the lesioned SN might not be because of cell replacement of dopaminergic neurons, but a homeostatic effect on the partially impaired nigrostriatal dopaminergic system. We postulate that trophic factors released by hM(N)SC stimulate an endogenous neuroprotective response in the lesioned SN. However, we cannot exclude other potential mechanisms, such as anti-inflammatory, antioxidant, and/or immunoregulatory actions [9,11,17,20,67].

In conclusion, the serum-free medium supplemented with EGF and bFGF used in this study leads to BDNF expression and delivery. Our protocol is simple and reproducible, and could thus be used in future cell therapies for several human neurodegenerative

disorders. In addition, the efficient intranigral engraftment of these epigenetically induced BDNF-secreting hMSC and the neurotrophic evidence observed after transplantation into the ongoing degenerated nigrostriatal dopaminergic system suggest that hM(N)SC hold the promise of being able to supply bioactive molecules beneficial for survival and maintenance of impaired dopaminergic neurons in PD. In this way, future therapies for several human BDNF-related neurodegenerative disorders could be approached using hMSC transplantation, taking advantage of their capacity for neurotrophic factor delivery.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bbmt.2010.06.006

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