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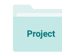

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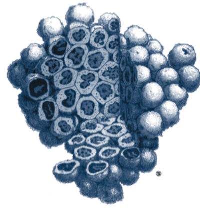

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Conversion of Human Umbilical Cord Mesenchymal Stem Cells in Wharton's Jelly to Dopaminergic Neurons In Vitro: Potential Therapeutic Application for Parkinsonism

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Key Words. Human umbilical mesenchymal stem cells • Parkinson's disease • Transplantation • Dopaminergic neuron • Tyrosine hydroxylase

ABSTRACT

Human mesenchymal stem cells isolated from Wharton's jelly of the umbilical cord were induced to transform into dopaminergic neurons in vitro through stepwise culturing in neuron-conditioned medium, sonic hedgehog, and FGF8. The success rate was 12.7%, as characterized by positive staining for tyrosine hydroxylase (TH), the rate-limiting catecholaminergic synthesizing enzyme, and dopamine being released into the culture medium. Transplantation of such cells into the striatum of rats previously made Parkinsonian by unilateral striatal lesioning with the dopaminergic neurotoxin 6-hydroxydopamine partially corrected the lesion-induced amphetamine-evoked rotation. Viability of the transplanted cells at least 4 months after transplantation was identified by positive TH staining and migration of 1.4 mm both rostrally and caudally. These results suggest that human umbilical mesenchymal stem cells have the potential for treatment of Parkinson's disease. STEM CELLS *2006; 24:115–124*

INTRODUCTION

Parkinson's disease is a neurodegenerative disorder characterized by the progressive loss of striatal dopaminergic function [1–6]. Patients initially respond to treatment with dopaminergic-enhancing medications such as levodopa [7]. However, the effectiveness of such treatments gradually diminishes because the conversion to dopamine within the brain is increasingly disrupted by the progressive degeneration of the dopaminergic terminals. As a result, after approximately 10 years of dopamine replacement treatment, most

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patients with Parkinson's disease suffer from disability that cannot be satisfactorily controlled [8].

An alternative approach for the restoration of the damaged dopaminergic system, considered to be an ultimate treatment of Parkinson's disease, is the transplantation of cells (or tissues) that synthesize catecholamines [9–13]. There is evidence both from animal studies and clinical investigations showing that fetal dopamine neurons can produce symptomatic relief [14–26]. However, technical and ethical difficulties in obtaining sufficient and appropriate graft tissues have limited the application of this therapy [27].

Mesenchymal cells from the Wharton's jelly of the umbilical cord possess stem cell properties [28–30]. We previously demonstrated that human umbilical mesenchymal stem cells (HUMSCs) could be induced to differentiate into neuron-like cells (approximately 87%), expressing neurofilament, functional mRNAs responsible for the syntheses of subunits of the kainate receptor and glutamate decarboxylase and generating an inward current in response to evocation by glutamate [29]. HUMSCs are also capable of differentiating into osteogenic, chondrogenic, adipogenic, and myogenic cells in vitro [30].

In the present study, HUMSCs were isolated and transformed into dopaminergic neurons in vitro. These dopaminergic neurons were then transplanted into the striatum of rats previously made Parkinsonian by unilateral striatal lesioning with 6-hydroxydopamine HCl (6-OHDA). The results indicated that transplantation of in vitro–differentiated HUMSCs alleviated the lesion-induced amphetamine-evoked rotation in the Parkinsonian rats, demonstrating potential therapeutic values.

Materials and Methods

Preparation of HUMSCs

Human umbilical cords were collected in Hanks' balanced salt solution (HBSS) (14185-052, Gibco, Grand Island, NY, http://www.invitrogen.com) at 4°C. After disinfection in 75% ethanol for 30 seconds, the umbilical cord vessels were cleared off while still in HBSS. The mesenchymal tissue (in Wharton's jelly) was then diced into cubes of approximately 0.5 cm^3 and centrifuged at 250*g* for 5 minutes. After removal of the supernatant fraction, the precipitate (mesenchymal tissue) was washed with serum-free Dulbecco's modified Eagle's medium (DMEM) (12100-046, Gibco) and centrifuged at 250*g* for 5 minutes. After aspiration of the supernatant fraction, the precipitate (mesenchymal tissue) was treated with collagenase at 37°C for 18 hours, washed, and further digested with 2.5% trypsin (15090-046, Gibco) at 37°C for 30 minutes. Fetal bovine serum (FBS) (SH30071.03, Hyclone, Logan, UT, http://www.hyclone.com) was then added to the mesenchymal tissue to neutralize the excess trypsin. The dissociated mesenchymal cells were further dispersed by treatment with 10% FBS-DMEM and counted under the microscope and with the aid of a hemocytometer. The mesenchymal cells were then used directly for cultures or stored in liquid nitrogen for later use.

Preparation of Neuronal Conditioned Medium

Seven-day postnatal Sprague-Dawley rats were anesthetized by intraperitoneal injection of 10% chloride hydrate. The brain was removed, placed in Ca^{2+}/Mg^{2+}-free buffer (14185-052, Gibco), and centrifuged at 900 rpm for 5 minutes. After removal of the supernatant fraction, 10% FBS-DMEM was added to the precipitate (brain tissue). The brain tissue suspension was triturated 15 times for dispersal into single cells. The cells were suspended in 10% FBS-DMEM and incubated at 37°C in 5% CO_2 and 95% O_2. To inhibit the growth of glial cells, 2 μM AraC (c-6645, Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) was added on the next day. On the fifth day of culture, the culture medium was removed (neuronal-conditioned medium [NCM]) to be used for the culture of umbilical mesenchymal cells. The HUMSCs were cultured in NCM alone, which was replaced every other day.

Generation of Tyrosine Hydroxylase–Positive Populations from Undifferentiated HUMSCs In Vitro

In vitro differentiation of HUMSCs into tyrosine hydroxylase–positive (TH^+) neurons was carried out as previously described [31], with modifications (Fig. 1). In stage 1, undifferentiated HUMSCs were dissociated into single cells and then cultured in

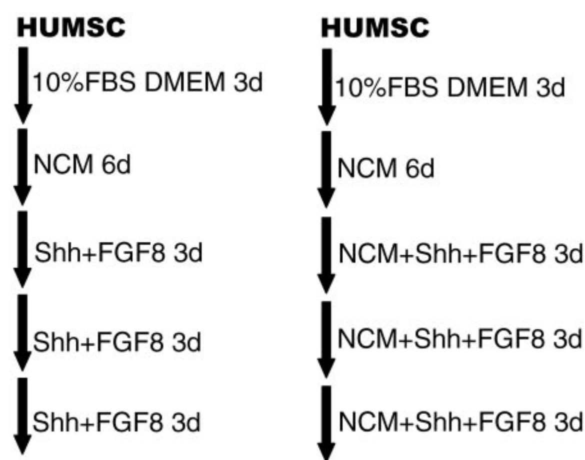

Figure 1. The grouping and scheme of in vitro incubation of HUMSCs. Tyrosine hydroxylase–positive populations were generated from undifferentiated HUMSCs by a three-step in vitro differentiation method. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGF, fibroblast growth factor; HUMSC, human umbilical mesenchymal stem cell; NCM, neuronal-conditioned medium; Shh, sonic hedgehog.

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10% FBS-DMEM for 3 to 6 days for expansion. In stage 2, HUMSCs were cultured in NCM alone for 6 to 9 days, which was replaced every other day to induce neuron-like differentiation. In stage 3, cells were supplemented with NCM or 10% FBS-DMEM in the presence of the murine *N*-terminal fragment of sonic hedgehog (SHH) (500 ng/ml, 461-SH, R&D Systems Inc., Minneapolis, http://www.rndsystems.com) and murine FGF8 isoform b (FGF8) (100 ng/ml, 423-F8, R&D Systems Inc.) for 3, 6, 9, or 12 days.

Immunocytochemistry for TH

HUMSCs and brain sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 minutes and washed with 0.1 M phosphate buffer. They were then treated with a blocking solution (0.05% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin) for 30 minutes to prevent nonspecific antibody-antigen binding. The cells and brain sections were then reacted with primary antibodies (mouse anti-TH, 1:333, MAB318, Chemicon, Temecula, CA, http://www.chemicon. com) at 4°C for 18 hours, washed with 0.1 M phosphate-buffered saline (PBS), reacted with secondary antibodies at room temperature for 1 hour, washed again with 0.1 M PBS, reacted with ABC complex (ABC KIT, PK-4000, Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) at room temperature for 1 hour, washed with 0.1 M PBS, and finally developed with 3,3′-diaminobenzidine (DAB) (5 mg DAB, 3.5 μl of 30% H_2O_2 in 10 ml of 50 mM Tris buffer).

Western Blotting for TH

Cell membranes were prepared from HUMSCs cultured in NCM, SHH, and FGF8 for varying periods. After resolution on 20% SDS-PAGE, the cell proteins were blotted onto polyvinylidene difluoride (PVDF) membranes, which were then washed with Tris buffer with 0.9% NaCl (pH 7.3), immersed in the blocking solution (0.05% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin) for 60 minutes, washed with TBS buffer again, and reacted with primary antibodies (mouse anti-TH, 1:333, MAB318, Chemicon) at 4°C for 12 to 18 hours. After the reaction was completed, the PVDF membranes were washed with Triton Tris buffer (0.05% Triton and 0.9% NaCl in 50 mM Tris-HCl, pH 7.3), immersed in the blocking solution for 60 minutes, and then reacted with secondary antibodies at room temperature for 1 hour. The PVDF membranes containing the reaction products were washed with Tris-buffered salt solution with Tween (TTBS), reacted with ABC complex (PK-4000, Vector Laboratories) at room temperature for 1 hour, washed again with TTBS, and finally developed with DAB.

Double Staining of Anti–Human-Specific Nuclear Antigen and Anti-TH, Anti-Dopamine-β-Hydroxylase, or Anti–Glutamate Decarboxylase

For the assessment of the possible differentiation of HUMSCs into subpopulations of dopaminergic, norepinephrine, or GABAergic neurons, we applied double staining for human-specific nuclear antigen [32] and TH, dopamine-β-hydroxylase (DBH), and glutamate decarboxylase (GAD).

HUMSCs and brain sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 minutes and then washed with 0.1 M phosphate buffer. They were then treated with a blocking solution for 30 minutes to prevent nonspecific antibody-antigen binding. The cells or brain sections were then reacted with primary antibodies (mouse anti–human-specific nuclear antigen, 1:25, MAB1281; mouse anti-TH, 1:333, MAB318; mouse anti-DBH, 1:1300, MAB308; rabbit anti-GAD, 1:1500, AB5992, all from Chemicon) at 4°C for 18 hours, washed with 0.1 M PBS, reacted with secondary antibodies (fluorescein-conjugated goat anti-mouse immunoglobulin G [IgG] for human nuclei, 1:50, AP124F; Rhodamine-conjugated goat anti-mouse IgG for TH or DBH, 1:50, AP124R; Rhodamine-conjugated goat anti-rabbit IgG for GAD, 1:50, 132R, all from Chemicon) at room temperature for 1 hour. The cells or brain sections were then observed under a fluorescence microscope.

Preparation of Parkinsonian Animals

Adult Sprague-Dawley rats (250 to 300 g) were used in this study. Under chloride hydrate anesthesia (400 mg/kg i.p.), the rats were placed in a stereotaxic frame. The dopamine-innervated striatum were unilaterally lesioned by administering injections of 6-OHDA into the median forebrain bundle AP: −4.3 mm, R/L: +1.6 mm, H: −8.2 mm and AP: −4.0 mm, R/L: +1.8 mm, H: −8.0 mm [33, 34]. Coordinates were set according to the atlas of Paxinos and Watson [35]. Each rat received 30 μg of 6-OHDA dissolved in 5 μl of physiological saline containing 0.02% ascorbic acid. Amphetamine-induced rotational behavior was assessed at 4, 8, 12, 16, and 20 weeks after 6-OHDA injection. For that, the rats were placed in individual plastic hemispherical bowls and allowed to habituate for 10 minutes before being injected with a subcutaneous dose of amphetamine (5 mg/kg). Left and right full-body turns were counted. Amphetamine-induced net rotation over a period of 60 minutes, starting 30 minutes after injection, was enumerated. Animals showing >360 turns per hour ipsilaterally toward the lesioned side after a single dose of amphetamine were considered successful Parkinsonian models and were selected for grafting [36–38]. All behavioral tests were performed in a closed room to avoid any environmental disturbance and assessed by an independent observer blind to the treatments.

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Experimental Grouping

The Sprague-Dawley rats were divided into three groups of 12 animals each. One month after 6-OHDA injection, rats in group 1 received PBS into their dopamine-denervated striata. Rats in group 2 received a suspension of 1×10^5 graft cells that had been cultured in NCM only, and rats in the third group received a suspension of 1×10^5 graft cells that had been cultured in NCM, SHH, and FGF8.

Preparation and Transplantation of HUMSC-Derived TH$^+$ Cells

The HUMSCs in stage 3 in vitro differentiation were treated with 1 μg/ml bis-Benzimide (B2883, Sigma-Aldrich) for 24 hours to label the cells. Cells were trypsinized at 37°C for 5 minutes with 0.25% trypsin, and the dissociated cells were resuspended in PBS. A total of 1×10^5 cells in a 10-μl suspension were transplanted into the striatum of each rat (anterior, 1.0 mm; lateral, 3.0 mm; ventral, −6.0 mm), based on positioning from the bregma and skull surface. A waiting period of 10 minutes before the needle was removed allowed the cells to settle. Rat hosts did not receive any immunosuppression medications.

Histological Examination of Grafted Brain Cryosections

For tracking of the transplanted cells, the cellular membrane penetrating and DNA-binding fluorescence probe bis-Benzimide was used. Twenty weeks after transplantation, the grafted rats were anesthetized terminally using an overdose of pentobarbital i.p., after which the brains were removed and postfixed for 24 hours in 4% paraformaldehyde in PBS at 4°C and then sectioned. Next, the specimens were equilibrated in 10% sucrose in PBS for 4 hours at 4°C, then in 15% sucrose in PBS for 4 hours at 4°C, and finally in 20% sucrose in PBS overnight at 4°C. They were then embedded in OCT compound and frozen in liquid nitrogen. Sections were cut into serial 30-μm-thick slices using a cryostat. The tissues were stained with the fluorescent stain bis-Benzimide and visualized under a fluorescence microscope for mapping of the stained cells.

High-Performance Liquid Chromatography Analysis of Dopamine Concentration

The culture medium was acidified with 0.1 N perchloric acid and centrifuged at 10,000*g* for 10 minutes. The supernatant was immediately frozen in liquid nitrogen and stored at −70°C until analysis. A high-performance liquid chromatography (HPLC) with endothelial cell detection procedure was used to quantify dopamine content in the supernatant [39]. Applied oxidizing potential was 0.75 V, and a 4.6- × 150-mm C18 column was used. The mobile phase contained 2.1 g heptanesulfonic acid, 0.1 g ethylenediamine-tetraacetic acid, 3 ml phosphoric acid, 3.5 ml triethylamine, and 130 ml acetonitrile in 850 ml deionized water. The retention time for dopamine was approximately 8 minutes.

Statistical Analyses

All data were presented as mean ± standard error. One-way or two-way analysis of variance was used to compare all means, and least-significance difference was used for the posteriori test. In all statistical analyses, $p < .05$ was considered significant.

Results

Acquisition of Dopaminergic Phenotype

Immunocytochemical staining for the catecholaminergic rate-limiting synthesizing enzyme TH showed that the HUMSCs were TH positive after incubation with NCM for 6 days and then SHH and FGF8 in 10% FBS-DMEM for 3, 6, or 9 days (Fig. 2A) or NCM for 6 days and then SHH and FGF8 in NCM for 3, 6, or 9 days (data not shown) but not after a 6-day NCM incubation only. Double staining of human-specific nuclear antigen and DBH and GAD indicated that the HUMSCs differentiated into, in addition to dopamine neurons, low yields of norepinephrine (Fig. 2B) and GABAergic neurons (Fig. 2C).

To facilitate counting cell number, the HUMSCs were cultured at a relatively low density (10^3 per ml). The proportion of cells expressing TH after treatment with NCM for 6 days followed by SHH and FGF8 for 3 days was 12.7% ± 2.1% ($p < .01$). No further increase in percentage of TH-containing cells was observed in cells treated with SHH and FGF8 for 6 or 9 days. No difference in the percentages of TH-expressing cells was observed between cells incubated in SHH and FGF8 in DMEM or NCM ($p > .05$) (Fig. 2D).

Human TH yields immunoreactive bands of 62 to 68 kD [40, 41], whereas that from the rat is estimated at 60 kD [42]. Our Western blot results showed that TH protein was not detected in HUMSCs treated with NCM only. The TH protein (68 kD) began to label significantly in the cells after treatment with NCM for 6 days and SHH and FGF8 for 3 or 6 days (Fig. 2E).

Dopamine was not detected in the medium of HUMSCs treated with DMEM or NCM. Dopamine concentration in the culture medium rose to a concentration of 51.0 ± 2.0 nM, as assayed by HPLC-ECD after a 6-day NCM and 3-day SHH and FGF8 culture of 10^5 cells in 10 ml of culture medium in a 100-mm-diameter culture dish (Fig. 2F) ($p < .01$).

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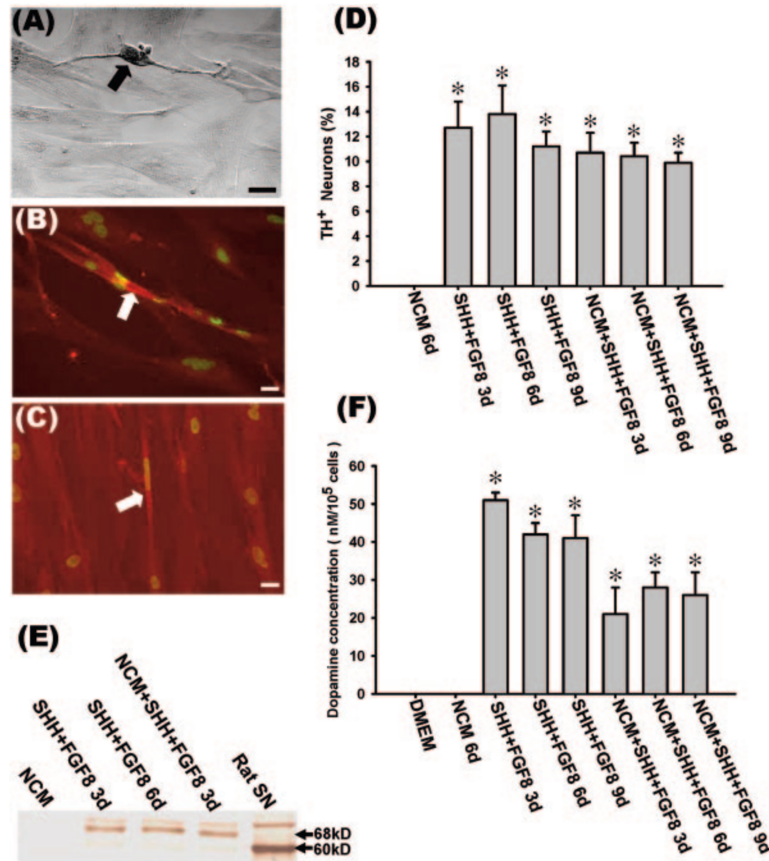

Figure 2. HUMSC differentiation into dopaminergic, norepinephrine, and GABAergic neurons in vitro. **(A):** Photomicrographs showing TH immunocytochemistry of cultured HUMSCs. The cells expressed TH after incubation with NCM for 6 days and then SHH and FGF8 in DMEM for 3 days. In addition to TH-positive neurons, DBH-positive **(B)** and GAD-positive **(C)** neurons were detected. Human-specific nuclear antigen are in green, and DBH and GAD are in red. Arrows indicate cells stained positively for TH, DBH, or GAD. Scale bar = 100 μm. **(D):** Histograms showing the percentage of TH-positive cells after incubation with NCM, SHH, and FGF8. (Results represent the mean ± standard error from three different experiments. At least 200 cells were counted from 10 randomly selected microscopic fields in each experiment. Statistics consisted of one-way ANOVA followed by the LSD test; *statistical difference at $p < .05$ compared with NCM-only group.) **(E):** TH expression in cultured cells by Western blotting. The molecular weight of rat and human TH were 60 and 68 kDa, respectively. Rat SN served as positive control. **(F):** Dopamine concentration in culture medium after HUMSCs were treated with NCM, SHH, and FGF8. (Results represent the mean ± standard error from three different experiments. Statistics consisted of one-way ANOVA followed by the LSD test; *statistical significance at $p < .05$ compared with DMEM and NCM-only groups.) Abbreviations: ANOVA, analysis of variance; DBH, dopamine-β-hydroxylase; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; LSD, least-significant difference; HUMSC, human umbilical mesenchymal stem cell; NCM, neuronal-conditioned medium; Shh, sonic hedgehog; SN, substantia nigra; TH, tyrosine hydroxylase.

TH Immunocytochemistry in Grafted Striatum

At 20 weeks after transplantation, bis-Benzimide–labeled cells were found in the striatum (Figs. 3A, 3B). Many cell somata staining positively for TH were clearly identified around the implantation site (Fig. 3C). Double-staining of human-specific nuclear antigen and TH indicated that the TH-positive cells were derived from HUMSCs (Fig. 3D). In contrast, no TH-positive soma was detected in the brains of rats that received grafted cells treated with NCM only (data not shown).

Cell migration patterns were followed by bis-Benzimide labeling in 30-μm serial sections. The labeled cells had migrated for approximately 1.4 mm in both directions of the rostrocaudal axis from the implantation site (Bregma +1.0). Most of the labeled cells were localized in the region of Bregma +2.0 to the region of Bregma −0.6, almost throughout the entire striatum (Fig. 4).

Effect of Transplantation on Amphetamine-Induced Rotation

The effects of stem cell transplantation were examined in 6-OHDA–lesioned animals by quantification of rotations in response to amphetamine [34–36]. Rotational scores were examined at 1, 2, 3, and 4 months after transplantation. One month after 6-OHDA lesioning, the number of amphetamine-induced

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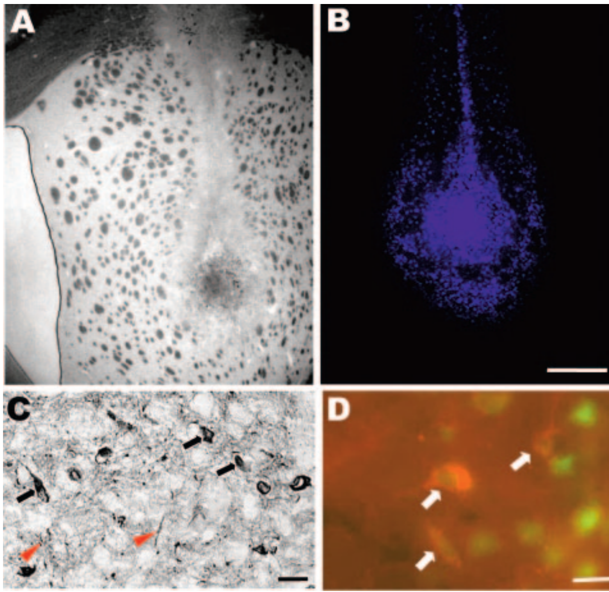

Figure 3. Photomicrographs showing the distribution of HUMSCs in rats 5 months after transplantation. The nuclei of HUMSCs were labeled with bis-Benzamide. The cells were microinjected into the striatum of Parkinsonian rats. The cells survived in the striatum 4 months after transplantation. Phase-contrast **(A)** and same-field **(B)** fluorescence photomicrograph. **(C):** Existence of TH-positive cell bodies in the grafted striatum. Arrows indicate cell bodies stained positively for TH. Arrowheads indicate processes stained positively for TH. **(D):** TH-immunoreactive cells doubly stained with anti–human-specific nuclear antigen in grafted striatum. Arrows indicate cells positively doubly stained for TH and human-specific nuclear antigen. TH staining is in red, and anti–human-specific nuclear antigen is in green. Scale bars = 1 mm **(B)** and 200 μm **(C, D)**. Abbreviations: HUMSC, human umbilical mesenchymal stem cell; TH, tyrosine hydroxylase.

rotations in all groups reached 381.0 ± 14.3 to 425.5 ± 19.7 rotations per hour with the control group ($n = 12$), which received injections of PBS in the dopamine-denervated striatum, showing a significant increase in numbers of rotation. Importantly, the increase in rotational scores was gradual over the months. No rats in the group that received grafted cells treated with NCM only ($n = 12$) showed any improvement ($p > .05$). All of the rats receiving grafted cells treated by NCM, SHH, and FGF8 rotated significantly less than those in the control and NCM groups at the first observation time (1 mo.). Such rotation, which did not continue to get worse like the control group, was consistently observed in the NCM + SHH + FGF8 group throughout the experimental period (Fig. 5, Table 1).

Discussion

Ideal donor cells for Parkinson's disease therapy should be easily available, capable of rapid expansion in culture, immunologically compatible, capable of long-term survival and integration in the host brain, and amenable to stable transfection and long-term expression of exogenous genes such as TH [43]. HUMSCs in Wharton's jelly of the umbilical cord can be easily obtained and processed compared with embryonic and bone marrow stem cells. In the present study, approximately 1×10^6 HUMSCs were collected from 20 cm of umbilical cord. The number of HUMSCs doubled (2×10^6) in 10% FBS-DMEM in 3 days. We also found that the transformed HUMSCs in the striatum were still viable 4 months after transplantation, without the need for immunological suppression, suggesting that HUMSCs might be a good stem cell source for transplantation.

The HUMSCs were induced to differentiate into TH^+ cells in vitro using a three-step protocol. The HUMSCs were expanded in 10% FBS-DMEM for 3 to 6 days in stage 1. In a previous study, similarly processed HUMSCs were found to express high levels of matrix receptors (CD44, CD105), integrin (CD29, CD51), and mesenchymal stem cell markers (SH2, SH3). Interestingly, these cells did not express hematopoietic lineage markers (CD34, CD45). These findings suggest that HUMSCs are similar to mesenchymal stem cells [30].

In this study, the HUMSCs were transformed into nondividing neurons after culturing in the NCM alone for 6 to 9 days in stage 2. Our previous studies showed that 59.4% ± 1.3% of the HUMSCs displayed robust immunostaining for neurofilament after 3 days of NCM treatment. The proportion of neurofilament-positive cells further increased to 87.4% ± 5.5%, reached a plateau on the sixth day, and persisted for up to 12 days after treatment. Double staining with BrdU and DAPI showed that most of HUMSCs were still able to proliferate in NCM for 3 days. On the ninth day of treatment with NCM, cell proliferation was no longer observed in most cells. At this stage, HUMSCs differentiate into neurons in the postmitosis phase [29]. In this study, the rats that received NCM + SHH + FGF8 cells did not develop any tumor in the brain, indicating the in vitro–prepared grafts did not contain a population of proliferating cells.

In the present study, the HUMSCs differentiated into dopaminergic neurons in 10% FBS-DMEM containing SHH (500 ng/ml) and FGF8 (100 ng/ml) in stage 3. Previous studies have demonstrated that the increase of TH-positive neurons was even more pronounced when SHH and FGF8 were applied to mouse neural stem cells during in vitro differentiation. For the transformation of signaling molecules to neural stem cells in the mouse, the combined treatment of SHH and FGF8 was the most effective inducer of dopaminergic neurons [44].

As for the concentration of SHH and FGF8, Lee et al. [31] used 500 ng/ml SHH and 100 ng/ml FGF8 in their study, whereas Perrier et al. [45] used 200 ng/ml SHH and 100 ng/ml FGF8. The concentrations of SHH and FGF8 used in our system were the higher ones.

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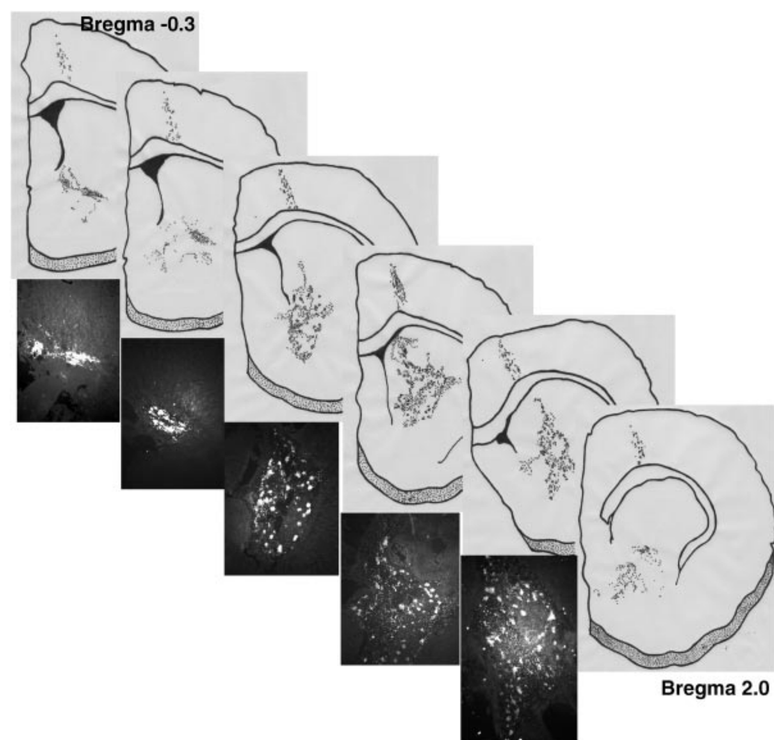

Figure 4. Line drawings of rat brain demonstrating the extent of human umbilical mesenchymal stem cell (HUMSC) migration after implantation in the striatum of the rat at the bregma level. ●, Cells of bis-Benzamide–labeled HUMSCs.

We used cells from a HUMSC-derived population as xenografts for unilaterally dopamine-denervated rats. Numerous studies have indicated that various growth factors, such as glial cell line–derived growth factor, transforming growth factor-beta, interleukin-1, or bone morphogeneic protein, are involved in the differentiation of embryonic cells into dopaminergic neurons [46–48]. In addition, SHH and FGF8 simultaneously induce the expression of dopamine-related proteins [31, 49]. Okabe et al. [49] and Lee et al. [31] reported a five-step in vitro differentiation method that yielded an efficient generation of dopamine neurons (33%) from undifferentiated mouse embryonic stem cells. We modified that protocol for the preparation of the graft cells used in the present study because the presence of dopamine neurons in the grafts and their subsequent production of dopamine are critical factors for the improvement of rotational behavior in Parkinsonian rats. We first examined whether the cells to be grafted expressed TH and produced dopamine. Bis-Benzidmide, used for tracking the cell movements, had no bearing on the percentage of TH-positive cells. Moreover, we detected TH protein production in the cells at stage 3 (Figs. 2A, 2D, 2E). Secreted dopamine was also detected by HPLC in the supernatant of cultured stage 3 cells (Fig. 2F). Therefore, we feel that differentiated cells from an undifferentiated HUMSC population can be used as grafts for the treatment of Parkinsonian rats. Although TH^+ cells only amounted to 12% of those in stage 3, we used whole fractions of the cells from that stage as grafts. Reasons that have been suggested to account for the difference in percentage yields include the species of stem cells and the different characteristics of embryonic stem cells and umbilical mesenchymal stem cells.

In our study, no increase in rotation that did not continue to get worse like the control (lesioned-only) group was observed 1 month after transplantation in all rats that received cells treated with NCM + SHH + FGF8. Subsequently, for the next 3 months, neither significant further improvements (reduction) nor deteriorations (elevation) were observed. In contrast, the rotation behavior in the NCM group was similar to that of the control (lesioned only) group, which continued to deteriorate with time, suggesting that undifferentiated neuronal cells derived from HUMSCs did not improve rotation behavior in Parkinsonian rats. The rotation in the NCM + SHH + FGF8 group was significantly decreased relative to the level of the control group, although not back to the normal level of intact rat. We suggest two possibilities. First of all, the number of dopaminergic neurons of implanted cells may have been relatively inadequate. Although it is difficult to determine the optimal number of dopamine neurons to be transplanted, we feel that the number of cells used in the present study ($12\% \times 1 \times 10^5$ cells per rat) was not enough to completely alleviate the Parkinsonism symptoms in the afflicted rats, as was the case after 33% of dopaminergic neurons from mouse embryo stem cells treated with SHH + FGF8 were transplanted into the striata of the Parkinsonian

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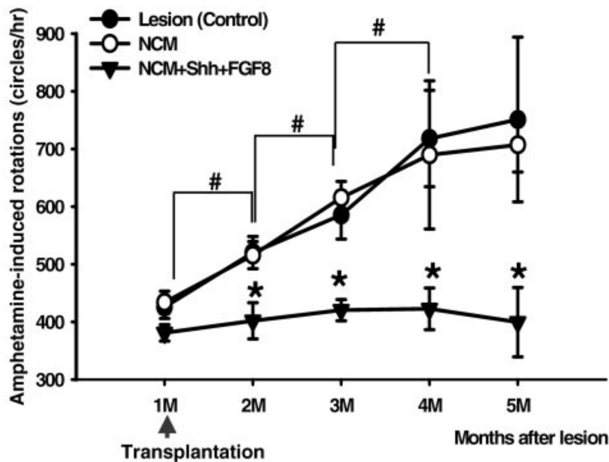

Figure 5. Rotation behavior in response to amphetamine tested at 1, 2, 3, 4, and 5 months after lesion. A significant decrease in the number of amphetamine-induced turning was seen in animals with grafted cells treated with NCM + SHH + FGF8 (▼, $n = 6$) compared with control (lesion-only) animals (●, $n = 12$) and lesioned animals that received grafted cells treated with NCM (○, $n = 12$). Statistics consisted of two-way ANOVA followed by the LSD test. (* Significant difference at $p < .05$ between NCM + SHH + FGF8-treated group compared with the control and NCM groups at the same time point. # Significant difference at $p < 0.05$ between the control and NCM groups over 1-month intervals.) Abbreviations: ANOVA, analysis of variance; FGF, fibroblast growth factor; LSD, least-significant difference; NCM, neuronal-conditioned medium; Shh, sonic hedgehog.

rats. There was significant improvement in the rotational behavior in the transplanted group compared with that in the lesioned group, although not to the extent of returning to the normal level, as in the case of transplantation of 80% of mouse embryo stem cells transfected with Nurr1 gene and treated with SHH and FGF8 for transformation into dopaminergic neurons [50]. We suggest that the number of dopaminergic neurons transplanted is an important factor in the treatment of Parkinson's disease. Similar results have been reported by Nishimura et al. [51].

Second, transplanted cells may take time to integrate in the host brain. The interaction time of donor cells may depend on the viability of transplanted cells and the species of the donor and the recipient. Studies have shown that symptomatic improvements can be observed by transplanting neurons from mouse, pig, and human embryonic brains into the rat brain [52–54].

Two rats in the NCM + SHH + FGF8 group survived for at least 8 months, with amphetamine-induced rotation behavior remaining similar to that 4 months after transplantation. The rest of the rats were euthanized for other experiments before 8 months. We plan to examine the long-term effects of transplantation.

Our findings may have a significant impact on the study of Parkinson's disease and potentially help to circumvent worrying ethical issues. Before human studies, we should first complete the observation of the effects and side-effects for longer than 1 year after transplantation, including behavioral effects, secretion of transmitters, activation of microglia, release of cytokines (such as tumor necrosis factor-α and interleukin-1_β), and pos-

Table 1. Rotations of individual rats in response to amphetamine tested at 1, 2, 3, 4, and 5 months after lesioning

Grouping	1	Rotation no. 1 month after lesion	2 months after lesion	3 months after lesion	4 months after lesion	5 months after lesion
Lesion	1	404	516	603	632	897
	2	397	528	616	789	854
	3	401	479	655	666	689
	4	386	459	503	799	876
	5	432	563	613	785	879
	6	442	511	546	713	736
	7	440	496	589	705	Die
	8	423	525	556	724	Die
	9	438	513	598	764	Die
	10	434	529	602	697	696
	11	423	506	587	603	587
	12	439	554	574	706	708
Lesion + NCM + SHH + FGF8	1	364	420	450	443	446
	2	403	433	401	351	339
	3	374	330	419	470	420
	4	404	432	421	409	411
	5	398	398	429	420	412
	6	392	421	405	422	352

Abbreviations: FGF, fibroblast growth factor; NCM, neuronal-conditioned medium; SHH, sonic hedgehog.

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sible development of brain tumor. Second, we should examine the toxicity included: growth factor (SHH and FGF8) and medium used.

Conclusion

We induced HUMSCs from Wharton's jelly of the umbilical cord to differentiate in vitro into a population that contained dopamine neurons using NCM, SHH, and FGF8. Transplantation of the HUMSCs partially corrected lesion-induced amphetamine-evoked rotational behavior. Our studies suggest the potential use of HUMSCs as therapy for Parkinson's disease.

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Disclosures

The authors indicate no potential conflicts of interest.

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