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Intrastriatal transplantation of mouse bone marrow-derived stem cells improves motor behavior in a mouse model of Parkinson's disease

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Summary Strategies of cell therapy for the treatment of Parkinson's disease (PD) are focused on replacing damaged neurons with cells to restore or improve function that is impaired due to cell population damage. In our studies, we used mesenchymal stromal cells (MSCs) from mouse bone marrow. Following our novel neuronal differentiation method, we found that the basic cellular phenotype changed to cells with neural morphology that express specific markers including those characteristic for dopaminergic neurons, such as tyrosine hydroxylase (TH). Intrastriatal transplantation of the differentiated MSCs in 6-hydroxydopamine-lesioned mice led to marked reduction in the amphetamine-induced rotations. Immunohistological analysis of the mice brains four months post transplantation, demonstrated that most of the transplanted cells survived in the striatum and expressed TH. Some of the TH positive cells migrated toward the substantia nigra. In conclusion, transplantation of bone marrow derived stem cells differentiated to dopaminergic-like cells, successfully improved behavior in an animal model of PD suggesting an accessible source of cells that may be used for auto-transplantation in patient with PD.

Keywords: Multipotent mesenchymal stromal cells (MSCs), dopamine, dopaminergic neurons, Parkinson's disease (PD), stem cells

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of over 50–60% of the dopaminergic neurons in the substantia nigra causing resting tremor, rigidity, bradykinesia and postural instability (Kish et al., 1988). Current drug therapy of PD by administration of dopamine (DA) precursors and agonists has many limitations and therefore the option of cell replacement therapy is constantly appealing (Lindval and Bjorklund, 2004; Winkler et al., 2005). However, largely negative results from previous controlled transplantation trials with fetal mesencephalic neurons in PD patients raise doubts about both the therapeutic benefit and disabling of such an approach in addition to safety and ethical concerns (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003).

Embryonic stem cells (ESc) may overcome the limitations of fetal donor tissue by offering both extensive cell proliferation and controlled differentiation to DA neurons (Kawasaki et al., 2000; Lee et al., 2000). Studies on ESc encouraged researchers to generate dopaminergic cells as an alternative source for transplantation in PD. Indeed, several groups reported that mouse and non-human ESc demonstrate dopaminergic characteristics following induced differentiation. Moreover, in subsequent experiments, transplantation in an animal model of PD demonstrated integration as well as behavioral recovery (Björklund et al., 2002; Kim et al., 2002; Barberi et al., 2003; Sanchez-Pernaute et al., 2005; Takagi et al., 2005; Kim et al., 2006). Recent work shows that human ESc might also differentiate into DA neurons, but the function of these cells has not yet been fully established (Ben-Hur et al., 2004; Zeng et al., 2004; Park et al., 2005; Yan et al., 2005; Brederlau et al., 2006; Roy et al., 2006). However, the poor survival of the ESc-derived TH positive cells following transplantation, in addition to the ethical and the safety issues, including teratoma formation, restrict the clinical usefulness of this type of stem cell (Carson et al., 2006, Roy at al., 2006).

We therefore focused on the induction of adult stem cells as an alternative. Multipotent mesenchymal stromal cells (MSCs) were previously reported to be multipotent and they can be induced *in vitro* to differentiate into a variety of tissues including osteoblasts, adipocytes, and chondrocytes (Prockop, 1997; Pittenger et al., 1999; Bianco and Robey, 2000; Bianco et al., 2001; Colter et al., 2000; Deans

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and Moseley, 2000; Krause, 2002). Additionally, recent findings including those from our laboratory indicate that mouse, rat and human MSCs can also be induced to differentiate into neuron-like cells (Sancez-Ramos et al., 2000; Woodbury et al., 2000; Levy et al., 2003; Blondheim, 2006). Moreover MSCs have the potential to migrate into injured neural tissues and differentiate into neurons (Mahmood et al., 2001; Munoz-Elias et al., 2004; Kan et al., 2005; Helman et al., 2006). Li et al. (2001) demonstrated that naïve mouse MSCs grafted into the striatum of mouse model of PD, promote some functional recovery at 28 days after transplantation. However, only about 0.8% of the grafted cells expressed tyrosine hydroxylase (TH).

In our previous studies, we demonstrated that mouse MSCs (mMSCs), exposed to an inducing cocktail, activated the neuron specific enolase (NSE) promoter and expressed typical neuronal markers (Levy et al., 2003). Moreover, most of the key gene for neuro-dopaminergic function are expressed in human MSCs (Blondheim, 2006). To examine whether bone marrow might be used for autologous cell replacement in PD, we isolated MSCs from enhanced green fluorescent protein (EGFP) transgenic mice, induced dopaminergic differentiation and transplanted the cells into a mouse model of PD. Our data indicate that differentiated mMSCs transplanted into the striatum of 6-OHDA-lesioned mice survive for several months, continually express neuronal markers and improve the amphetamine-induced rotational behavior.

Material and methods

Animals

Primary culture of mMSCs were obtained from adult B5/EGFP transgenic (Tg) mice bearing the *EGFP* gene and expressing the EGFP protein in all the tissues (Hadjantonakis et al., 1998). The behavior of mutant cells can be followed with simple ultra violet (UV) microscopic observation. The Tg mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). C57/b1 male mice (~30 gr) (Harlan Lab, Israel) were used for 6-OHDA lesions. All animals were housed in standard conditions: constant temperature ($22 \pm 1^{\circ}C$), humidity (relative, 50%), 12-h light, 12-h dark cycle and free access to food and water. All the animal experiments were performed under the supervision of the Animal Care Committee and Experimentation of The Faculty of Medicine at Tel Aviv University and at the Rabin Medical Center, Israel.

Isolation and culture of mMSCs

Mouse MSCs were isolated and cultured as described in our previous report (Levy et al., 2003). Briefly, cells were extracted from tibia and femur bones and placed in Hank's balanced salt solution (HBSS; Biological Industries, Bet-Haemek, Israel), centrifuged and plated in growth medium containing Dulbecco's Modified Eagle's Medium (DMEM; Biological Industries) supplemented with 15% fetal calf serum (FCS; Biological Industries), 5% horse serum (HS; Biological Industries), 1× nonessential amino acid (Biological Industries), 0.001% β-mercaptoethanol (Sigam, St. Louis, MO, USA), 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 12.5 units/ml nystatin (SPN; Biological Industries, Israel), in polystyrene plastic tissue cultures 75 cm^2 flask (Corning Incorporated, Corning, NY, USA), maintained at 37°C in an humidified 5% CO_2 incubator. Cells were incubated for 48 h then non-adherent cells were removed. The tightly adhered mMSCs cells were washed twice with Dulbecco's phosphate buffered saline (PBS; Biological Industries, Israel) and fresh growth medium was added. The medium was replaced every 3 or 4 days and when cells reached 70–90% confluency, cultures were harvested with trypsin-EDTA solution (0.25% trypsin and EDTA 1:2000 in puck's saline; Biological Industries, Israel) for 5 min at 37°C.

Flow cytometry analysis (FACS)

Following thirty days in culture, the isolated mMSCs were harvested from the tissue culture flasks. The cells (0.5×10^6) were stained for 45 min at 4°C with anti CD45 (1:200, eBioscience, San Diego, USA), anti CD90 (1:20, Miltenyi Biotec, Auburn, CA, USA), anti CD106 (VCAM-1) (1:400, BioLegend, San Diego, USA) conjugated to FITC or PE. Isotype control staining was performed with IgG2b-FITC (1:200, eBioscience) and IgG2b-PE (1:200, eBioscience). The labeled cells were thoroughly washed twice in flow-buffer (5% FCS, 0.1% sodium-azid in PBS).

The intracellular detection was performed as described previously (Hamann et al., 1997). Cells were fixed with 4% paraformaldehyde (Sigam) in PBS. Fixation was followed by permeabilization with 0.1% saponin (Sigma/Fluka), 10% goat serum (Biological Industries, Israel) in PBS at 4°C for 10 min. Washing buffer containing 0.1% saponin, 0.5% bovine serum albumin (Sigma) in PBS was used for all subsequent incubation and washing steps. Cells were stained with anti neurofilament heavy 200 (NF-200; Sigma, 1:100) primary antibody. They were incubated for 30 min at room temperature (RT) and followed by second antibody conjugated with Alexa 488 (Molecular-Probes, Oregon, USA, 1:500). Control staining was performed only with the secondary antibody.

Cells were resuspended in 0.5 ml PBS and studied by a FACSCalibur™ flow cytometer using an argon ion laser, adjusted to an excitation wavelength of 488 nm (FACS; Becton Dickinson Immunocytometry System, San Jose, CA, USA). Data was acquired and analyzed by CELLQuest™ version 3.0 software (Becton Dickinson). A minimum of 10,000 events were examined per sample. A non-specific isotype control was included in each experiment, and specific staining was measured from the cross point of the isotype with the specific antibody graph. Each value is the mean ± S.E. if more than two independent experiments were involved.

Differentiation to adipocytes

Adipogenic differentiation was induced and cells were stained following detailed protocols by Peister et al. (2004). Briefly, the mMSCs were incubated in DMEM that was supplemented with 10% FCS, 10% HS, SPN, 12 mM L-glutamine, 5μg/mL insulin (Sigma), 50 μM indomethacin (Sigma), 1 μM dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). The medium was changed 2 times per week for 3 weeks. Cells were fixed with 10% formalin for 20 min at RT and stained with 0.5% Oil Red O (Sigma) in methanol (Sigma) for 20 min at RT. Adipogenic differentiation was identified by Oil Red O staining of lipid vacuoles, seen as bright red inclusions within the cells.

Differentiation to neuron-like cells

mMSCs were cultured for at least 14 days, as described above. To induce differentiation to neuron-like cells we used our previously described protocol with some modifications (Levy et al., 2003; Blondheim et al., 2006).

Growth medium was replaced with Differentiation Medium I consisting of DMEM supplemented with 10% FCS, 2 mM glutamine, SPN, 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA), 10 ng/ml epidermal growth factor (EGF; R&D Systems), and N2 supplement (5μg/ml insulin; 20 nM progesterone; 100 μM putrescine; 30 nM selenium; 100 μg/ml transferring; all from Sigma) (Bottenstein, 1985), for 24–48 h. Differentiation media-I was then removed and cells were washed with PBS and transferred to Differentiation Medium II, composed of DMEM supplement with 2 mM glutamine, SPN, N2 supplement, 200 μM butylated hydroxyanisole (BHA; Sigma, Israel), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), and 1 μM all-trans-retinoic acid (RA; Sigma) for 48–72 h.

Western blot analysis

Protein extracts from mMSCs and neuron-like cells from mMSCs were prepared in 50 μl of cold buffer containing 105 mM Tris (Sigma), 5 mM EDTA (BDH Laboratory Supplies, Poole, UK), 140 mM NaCl (BioLab, Jerusalem, Israel), 10 mM sodium fluoride (Sigma), 0.5% NP-40 (United States Biochemical Corporation, Cleveland, OH, USA), 1 μM PMSF (Sigma). Homogenates were centrifuged at $13000 \times g$ for 20 min at 4°C, and supernatants were collected. Protein concentration was determined and 50 μg samples diluted 1:5 with sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% Glycerol, 2% sodium dodecyl sulfate, 5% 2-β-mercaptoethanol, 0.0025% bromophenol blue, Sigma) and boiled for 5 min heated prior to loading. Proteins were size fractionated on 12.5% SDS-polyacrylamide gels and electroblot transferred to polyvinylidene difluride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with primary antibodies mouse anti TH (1:10000, Sigma), and actin (1:1000, Chemicon, Temecula, CA, USA) was used to evaluate and quantify the changes during the induction. Membranes were then exposed to horseradish-peroxidase conjugated goat anti-rabbit IgG diluted at 1:25000, or anti-mouse IgG diluted at 1:20000 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), for 30 min at room temperature. The membranes were then stained using the enhanced SuperSignal® chemiluminescent detection kit (Pierce, Rockford, IL, USA) and exposed to medical X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometry of the specific proteins bands was preformed by VersaDoc® imaging system and Quantity One® software (Bio-Rad Laboratories).

Immunocytochemistry

mMSCS were plated and treated in slides chamber (Nalge Nunc International, Napervilee, IL, USA) previously treated with poly-L-lysine (Sigma). Cells were fixed with 4% paraformaldehyde (Sigma) and blocked with 0.1% Triton X-100 (Sigma) and 10% goat serum (Biological Industries, Israel) in PBS. The differentiated mMSCs were stained with the mouse antibodies against TH (1:2000 Sigma), neuronal nuclei antigen: Neu-N (1:40, Chemicon). Appropriate Cy3-labeled secondary antibodies (1:400 v/v; Jackson ImmunoResearch Laboratories) were used for visualization.

6-Hydroxydopamine lesion in mice

c57/bl male mice (30 gr) were anesthetized with chloral hydrate 350 mg/kg intra-peritoneally (i.p.) and secured in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Mice were unilaterally injected with 6-OHDA hydrobromide (4 μg in 2 μl saline with 0.01% ascorbate, Sigma) using a Hamilton 10 μl syringe with a 26-gauge needle (Hamilton, Reno, NV, USA), into the right striatum, at rate of 1 μl/min. The coordinates of the injections into the striatum were as follows: anterior 1.1 mm, lateral 2.3 mm, dorsa ventral 4.2 mm, with respect to bregma, based on the mice Stereotaxis Atlas (Paxinos and Franklin, 2001). At the completion of the injection, the needle was left in place for another 3-min period and then withdrawn at 1 mm/min in order to prevent a vacuum. Lesioned mice were tested for ipsiversive rotational behavior induced by an intraperitoneal (i.p.) injection of amphetamine (10 mg/kg; Sigma) 14 days after the 6-OHDA lesion. This test is widely used as a reliable index of dopamine depletion in the striatum (Hefti et al., 1982; Carman et al., 1991; Hudson et al., 1993; Thomas et al., 1994; Pavon et al., 1998). The clockwise turnings of each animal were measured visually, in turn, in a round tool for a period of 30 min. Only mice with a rotation rate of above 160 turns per 30 min were considered to be an established PD model and were used later for the grafting and control experiments.

Cell transplantation

Three weeks after the 6-OHDA lesion, the mice were divided into four experimental groups: saline, fibroblasts, mMSCs and differentiated mMSCs ($n = 5$). Saline or 2×10^5 vital cells/2μl were stereotactically injected into the lesioned striatum using a stereotaxic frame (anterior 1.1 mm, lateral 2.3 mm, dorsa ventral 4.2 mm, with respect to bregma). The rotational behavior was measured for 30 min, 30 min following amphetamine injection (i.p. 10 mg/kg).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Jackson-Lewis and Liberatore, 2000) with some modification. Briefly, at the end of treatment, mice were anaesthetized with chloral hydrate (350 mg/kg), then perfused transcardially with 20 mL of saline for 3 min followed by 80 mL of 4% paraformaldehyde in 0.1 M sodium-phosphate buffer (pH 7.1). Brains were removed, fixed for 72 h at 4°C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 2 days at 4°C. The brains were frozen by immersion in dry ice-cooled 2-methylbutane and stored at −70°C until sectioned. For each mouse, cryostat-cut sections (20 μm) throughout the entire ventral midbrain were collected free floating and adjacent sections were stained for TH. Briefly, sections, were first rinsed (3×5 min) with 0.1 m PBS (pH 7.4), followed by incubation with 5% normal goat serum (NGS; Biological Industries) for 60 min. Sections were then incubated on a shaker with the primary antibody rat anti-TH (1:2000, v/v; Calbiochem, San Diego, CA) in PBS, containing 2% NGS and 0.3% Triton X-100, for 48 h at 4°C. After rinsing in PBS, secondary antibody donkey anti rabbit conjugated to AMCA or Cy3 (1:100 v/v; Jackson ImmunoResearch Laboratories) in PBS, pH 7.4, containing 2% NGS was added and the sections were incubated for 60 min at room temperature. Transplanted cells were identified by immunostaining using goat anti-EGFP antibodies (1:2000 v/v; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by second antibody donkey anti goat conjugated to Cy2 (1:100 v/v; Jackson ImmunoResearch Laboratories). All sections were then washed for 3×5 min in PBS, Sections were coverslipped with fluorescence mounting medium (DAKO, Denmark).

Microscopy and image analysis technique

An Olympus BX52TF microscope was used to analyzed slides for histopathology. ViewfinderLite™ software, with a DP50 microscope digital camera system attached to the microscopes, was used to acquire images and the StudioLite™ software was used to edit and analyze recorded images (Olympus, Tokyo, Japan).

Image analysis was performed on four representative areas of each slide using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD, USA). The analysis of brain slices used to quantify the entire area of the striatum, ventricle, thalamic nucleus nigrostriatal bundle, ventral tegmental, medial globus pallidus, medial forbrain bundle, internal capsule and

substantia nigra, was photographed in a series of nine frames (a total of 45 pictures for each brain).

Dopamine content analysis

Hemispheric dopamine (DA) levels were determined in the 6-OHDA lesioned mice. Each hemisphere was homogenized in 1 ml of 0.1 N perchloric acid (Sigma) and centrifuged at $15000 \times g$ for 15 min at 4°C. The supernatant was filtered through a nylon filter (COSTAR, Spin X HPLC, 0.22 µm; Corning, NY, USA). An aliquot of the filtrate was injected into a high performance liquid chromatography system with an electrochemical detector (HPLC-ECD) (LC-4B and TL-5A, Bioanalytical Systems, West Lafayette, IN, USA) equipped with a C18, reverse phase column (125 mm × 4.6 mm) (Hichrom, Berkshire, UK). The sample was eluted by a mobile phase made of a 150 mM monochloroacetate buffer (pH 3) containing 10% methanol, 30 mg/L sodium 1-octanesulfonate, and 2 mM EDTA at flow rate of 1.2 ml/min. DA peak was determined by electrochemical detection at a potential of 650–700 mV. DA was identified by retention time and was validated by co-elution with catecholamine standards under varying buffer conditions and detector settings.

Statistical analysis

All data presented as means ± standard error of the mean (SEM). Significance of the differences between the rotational behavioral data following amphetamine administration was analyzed by the one-way Anova (SPSS, version 11.5) in order to analyze the data presented in Fig. 5. All the *in vitro* experiments were performed at least twice, in triplicate, and a representative figure is shown. In all tests, significance was assigned when $P < 0.05$.

Results

Isolation and culture of EGFP-Tg mice MSCs

Mouse bone marrow cells were isolated from tibias and femurs bones of EGFP-Tg mice and were plated in a growth medium. The cells were incubated for two days and non-adherent cells were removed. The plastic-adherent cells from EGFP-Tg mice bone marrow, divided and grew to 80–90% confluency within about 20 days. During this culture period, the cells expanded from a few cells in the flask to 1.5×10^6, demonstrating the homogeneous morphology of mesenchymal cells, a typical spindle-like cell morphology (Fig. 1A).

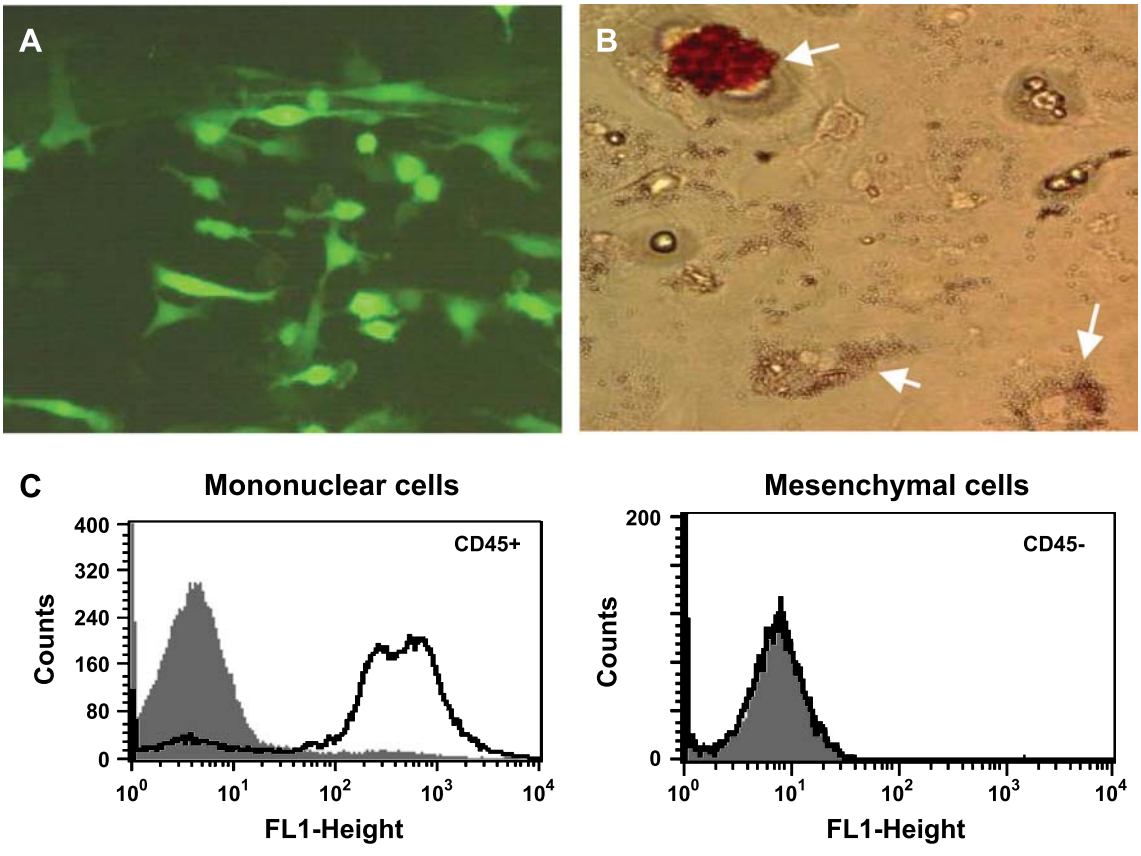

Fig. 1. Characterization of mouse multipotent mesenchymal stromal cells (mMSCs). **A** Isolated mouse mMSCs from EGFP transgenic mice. **B** mMSCs following adipogenic differentiation were stained by Oil Red O to detect lipid vacuole production by the cells (bright red inclusions within the cells). **C** Comparison of FACS analysis for CD45 of bone marrow derived mononuclear cells (left panel), with the plastic adherent cell population cultured *in vitro* over two weeks (right panel). After two weeks *in vitro*, a significant area of staining was found for hematopoietic markers CD45. Emphasized lines represent staining for non-specific IgG isotype fluorescence used as a control. Quantitative measurements were made from the cross point of the IgG isotype graph with the specific antibody graph

The cultured cells displayed further traits of MSCs, including the capability of readily differentiating into adipocytes as indicated by Oil-Red O staining when exposed to appropriate differentiation conditions (Fig. 1B).

The percentage of cells expressing mesenchymal markers significantly increased from day 0 to 20 and onward indicated by flow cytometry analysis. The cultured cells were positive for CD106 (27%) and CD90 (20%), typical mouse mesenchymal markers, and negative (<5%) for the hematopoetic marker CD45. In contrast, mononuclear cells in the bone marrow aspirate were positive to CD45 (80%, Fig. 1C). Although the mesenchymal cells isolated from different mice strains differ in their profile of surface markers, they share the same capacity to adhere to the plastic, show typical morphology and have a similar differentiation potential which indicate their identity as mesenchymal stromal cells (Peister et al., 2004; Dominici et al., 2006).

Differentiation of mMSCs to neuron-like cells

Following the 20 day of culture we induced neuronal differentiation by a two-step procedure. Firstly, cells were grown for 48 h in medium supplied with bFGF, EGF and N2 reagents. Secondly, cells were incubated with the serum free medium that contained BHA, RA, N2 supplement, and elevated cAMP for another 48 h, similar to the protocol we previously described for human MSCs (Levy et al., 2003; Blondheim et al., 2006; Hellmann et al., 2006). Microscope analysis indicated that during the induction of differentiation cells developed typical neural-like structures resembling dipolar cells, retractile cell bodies, neurites and axons, and long branching processes with growth cone-like terminal structures. The changes could be detected 24 h following the addition of the differentiation media and were sustained for three days (Fig. 2A). In contrast, cells grown in the expanded growth medium, exhibited the typical flat fibroblast-like morphology.

The neuronal-like morphological changes were accompanied by positive immunoreactivity for a typical neuronal marker. As indicated by FACS analysis, most cells exhibiting neuronal morphologies expressed neurofilament-200 (NF-200), a filament protein present in the axons and the synaptic terminals (Fig. 2B); neuron specific enolase (NSE), an isozyme of the glycolytic enzyme enolase expressed in

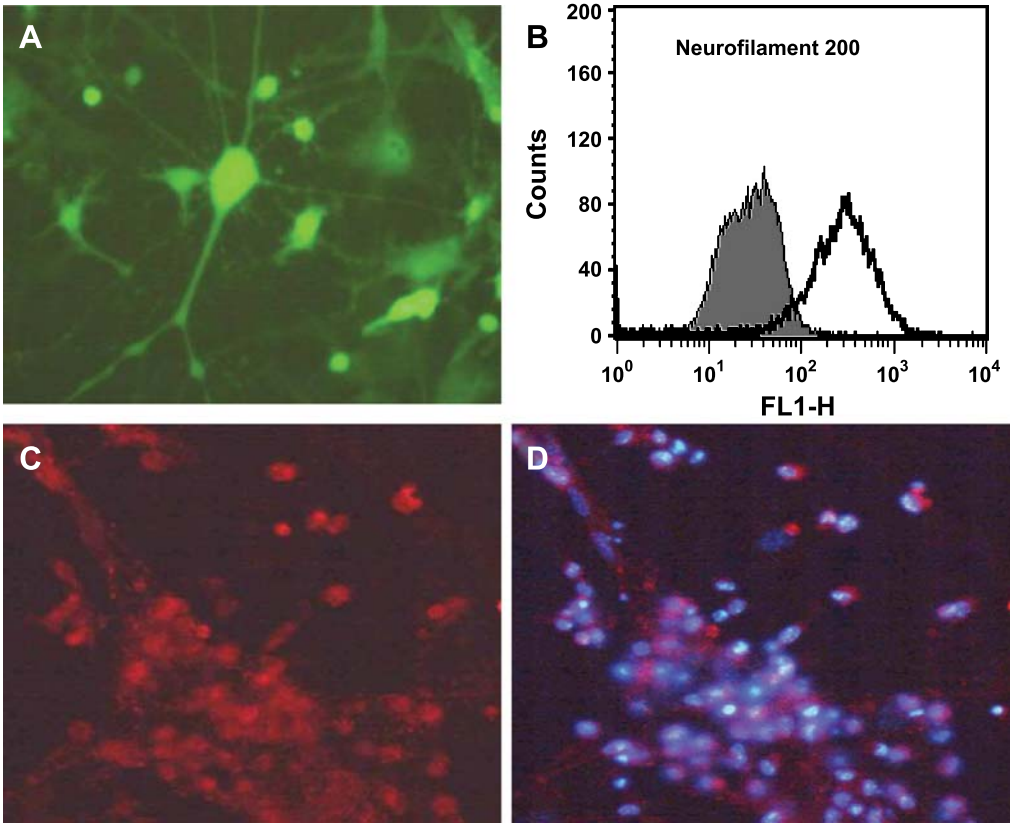

Fig. 2. Mouse multipotent mesenchymal stromal cells differentiate into neuron-like cells. Bone marrow cells were isolated from the femur and tibia bones of EGFP-Tg and differentiated to neuron-like cells. **A** Adherent cells after induction of differentiation. **B** FACS analysis for neurofilament-200. **C** Immunocytochemistry analysis with anti-NeuN antibodies and **D** nuclear DNA staining with DAPI

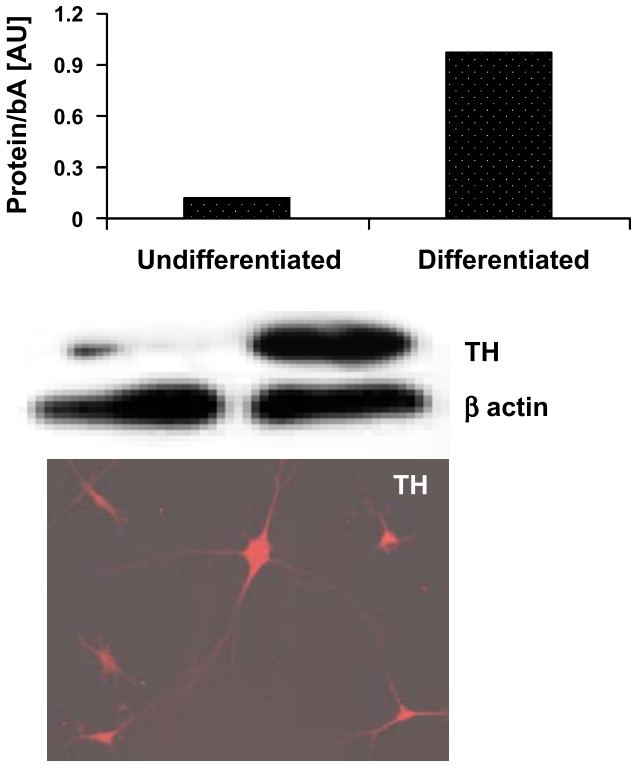

Fig. 3. Differentiated mouse multipotent mesenchymal stromal cells express tyrosine hydroxylase (*TH*). After 48 h of differentiation anti-TH were used for immunocytochemistry and Western blot analysis. The TH immunoreactivity and densitometry was normalized to the reaction with anti-beta-actin antibodies

all neuronal cell types (data not shown). In addition, the expression of the neuronal nuclei antigen Neu-N protein was markedly increased following the differentiation induction, as seen by immunohistochemistry (Fig. 2C, D).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of dopamine and a marker of ventral midbrain neurons. Furthermore, the differentiation induced a dramatic increase in tyrosine hydroxylase (TH) protein expression, as indicated by immunocytochemistry and Western blot analysis (Fig. 3).

Intrastriatal cell transplantation in a mouse model of PD model

To test whether the differentiated mMSCs might be beneficial following brain transplantation, we generated mice with DA-neuronal damage as an animal model of PD. As seen in Fig. 4, intrastriatal injection of 6-OHDA induced lesion and decreased the number of the TH positive cells in the substantia nigra. Accordingly, dopamine concentration was reduced by 50%, which indicates relatively moderate damage. The 6-OHDA lesioned mice emphasized the typical rotational-behavior induced by amphetamine.

Three weeks post 6-OHDA injection, MSCs and DA-differentiated cells, taken from EGFP-Tg mouse, were injected into the lesion striatum. The control groups included transplantation with non-differentiated mMSCs cartilage-derived ear fibroblasts and saline. During the three month experiment, amphetamine-induced rotations were compared to the number of rotations before the saline injection or cell transplantation of each mouse. The rotational behavioral was measured 2, 4, 6, 8, 12 weeks post engraftment for fibroblast cells and mMSCs group, or 3, 5, 8, 10, 11 weeks post engraftment for saline and differentiated mMSCs group. In the saline-injected group ($n = 5$), there were no changes and the mice demonstrated $92–106 \pm 8.7\%$, of the initial rotations during the experimental period (Fig. 5). The group of mice with engrafted fibroblast cells ($n = 5$) demonstrated stable rotational behavior for 12 weeks with no significant change (Fig. 5). By contrast, a moderate reduction of about 50% ($p < 0.05$) was seen after transplantation of EGFP non-differentiated mMSCs ($53 \pm 15\%$) (Fig. 5). However, transplantation of the DA-differentiated mMSCs cells demonstrated a marked reduction in the rotational behavior which peaked after 11 weeks by which time mice did not rotate at all following the amphetamine challenge ($13.5 \pm 8.5\%$, $p < 0.001$ vs. saline, Fig. 5).

Histological analysis was performed on the sacrificed mice at the end of the experiment (12 weeks). Most of the EGFP-positive transplanted cells were located in the striatum, around the injected area (Fig. 6). Surprisingly, few of the transplanted cells migrated to the neighboring areas along the dopaminergic track, whereas cells were observed in the nigrostriatal bundle, ventral tegmental, medial globus pallidus, medial forbrain bundle and internal capsule. Double immunostaining in the striatum revealed that some of the EGFP-positive transplanted cells were also TH positive, indicating a continuous stable expression of the dopaminergic marker 12 weeks post-transplantation (Fig. 6). Indeed, we found that low, but significant amount of cells migrated toward the substantia nigra indicated the presence of TH positive bone marrow derived EGFP cells adjacent to the endogenous dopaminergic cells.

Discussion

In the present study we further examined the differentiation induction of mMSCs into neuron-like cells. The mouse multipotent mesenchymal stromal cells underwent similar neural differentiation processes with minor modification to those noted in our previous studies using human MSCs

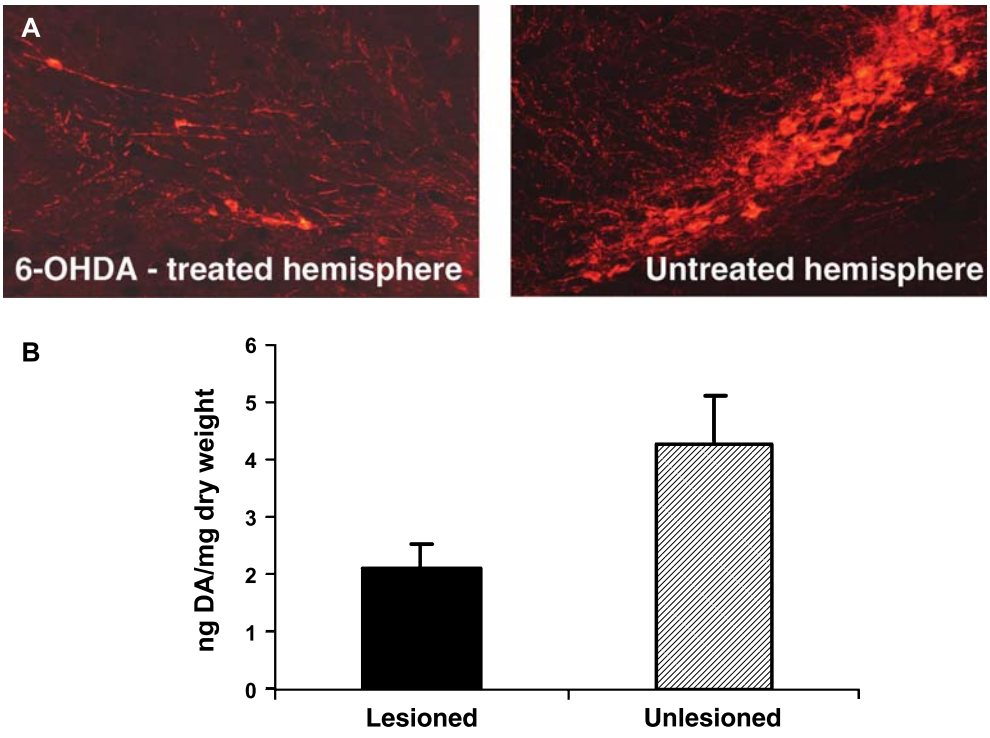

Fig. 4. Intrastriatal injection of 6-OHDA reduces the number of tyrosine hydroxylase (TH) positive neurons in the ipsilateral substantia nigra (SN). **A** Loss of dopaminergic cell bodies in the SN was achieved by injection of 6-OHDA to the mouse striatum as indicated by anti-TH staining. **B** The dopamine level in the hemisphere extract as measured by HPLC

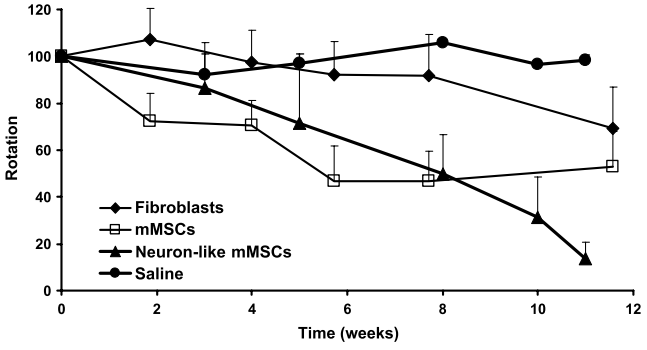

Fig. 5. Intrastriatal transplantation of differentiated multipotent mesenchymal stromal cells in 6-OHDA lesioned mice reduces amphetamine-induced rotational behavior. Three weeks after 6-OHDA injection, saline, fibroblasts, mMSCs and differentiated mMSCs (2×10^5 cells, n = 5). Rotational behavior was measured 30 min following amphetamine challenge, for a period of 30 min

(Levy et al., 2003, 2004; Blondheim et al., 2006; Kan et al., 2007). After three weeks in culture, the plastic adherent mMSCs expressed CD106 and CD90, which are known as mouse markers for mesenchymal cells (Baddoo et al., 2003; Peister et al., 2004). Although most of the fresh bone marrow contained a high percent of hematopoietic stem cells, the mMSCs subpopulation did not express the hematopoietic marker CD45. In addition, the cells were capable of differentiating into adipocytes, and demonstrating their characteristics.

Following neural differentiation protocol, cells changed their morphology from fibrocystic-like cells to a neuronal-like morphology including bipolar and long process formation. We also followed the expression of several neuronal specific markers such as Neu-N, NSE, NF-200 and found a marked increase in their presence during the three days of differentiation induction. Most interestingly, TH, the key enzyme for dopamine synthesis, was dramatically elevated, indicating that the cells differentiated into the dopaminergic pathway.

The latter assumption was further examined *in vivo*, using the unilateral instrastriatal 6-OHDA mouse model of PD. We have chosen to use mice to allow allogenic engraftment with $EGFP^+$ mMSCs. Unilateral 6-OHDA injections into the striatum caused a marked decrease in striatal TH^+ immunostaining terminals and also in the dopamine levels, as indicated by HPLC. The lesions also induced a rotational behavior after amphetamine challenge. This mouse 6-OHDA model has been used in several studies and it was shown that both the terminals and the cell bodies were damaged although not all of the nigral dopminergic cells

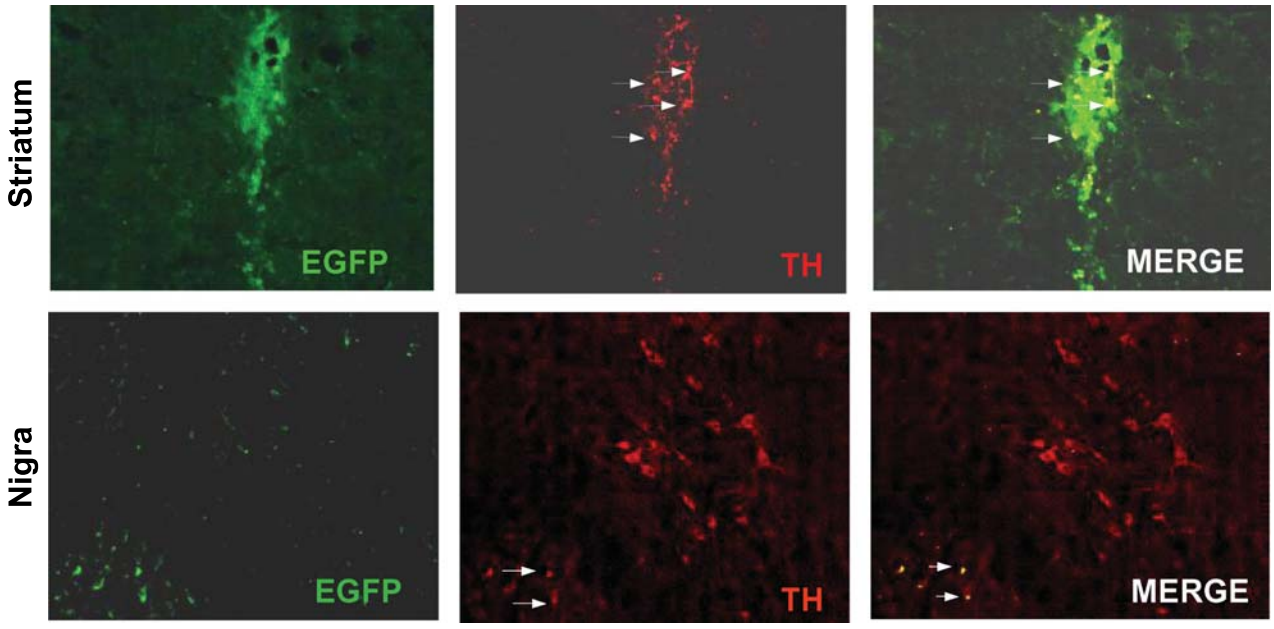

Fig. 6. Survival and migration of engrafted differentiated multipotent mesenchymal stromal cells in the injected hemisphere. Immunohistochemistry analysis using anti-tyrosine hydroxylase (TH) and anti-EGFP antibodies revealed the presence of TH-expressing cells among the transplanted EGFP-positive cells in the injection site (superior panel). TH^+-EGFP cells are also seen in the substantia nigra, probably after migration (lower panel)

disappear (Akerud et al., 2001). Therefore, recovery of the dopaminergic system should include replacement of the dopaminergic neuronal loss.

As we described previously (Levy et al., 2004; Blondheim et al., 2006), exposure to N2 supplement, BHA, RA, and an elevated of intracellular cAMP level, direct the MSCs to neuronal differentiation in human MSCs. In the present study we used mouse MSCs for transplantation in mouse model of PD. We observed a significant improvement two weeks following transplantation of the DA-differentiated MSCs, which constantly increase during the experiment and show almost full recovery after 11 weeks. The (non-differentiated) MSCs also show benefit six weeks post transplantation, than the rotational behavior was stable demonstrating no further improvement.

The capability of MSCs to protect and even regenerate affected neurons have been reported in various animal models of neurodegenerative diseases, such as multiple sclerosis (Zhang et al., 2005, 2006), amyotrophic lateral sclerosis (Mazzini et al., 2006) and stroke (Mahmood et al., 2005; Seyfried et al., 2006; Shen et al., 2007).

Two previous studies demonstrated some improvement of MSCs grafted into model of PD. The first, Li et al. (2001) grafted undifferentiated mMSCs into the striatum of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. The grafted MPTP-treated mice exhibited significant improvement on the rotarod test at 35 days after transplant, compared to nongrafted controls. However, only ~0.8% of the implanted cells expressed TH immunoreactivity. The second, Dezawa et al. (2004) showed motor improvement in a rat model of PD following intrastriatal implantation of MSCs transfected with Notch intracellular domain (NICD).

Indeed, it has already been shown in rodents and monkeys and even in some parkinsonian patients that replacement and significant reduction in the symptoms can be achieved with a relatively low number of engrafted dopaminergic cells (Studer et al., 1998; Takagi et al., 2005; Piccini et al., 1999). However, our study is the first time that neuronal-like cells differentiated from MSCs, without artificial gene overexertion, exhibited a long period of viable engraftment, cell survival and demonstrated improvement in the PD model. Further investigation is required to understand the mechanism of recovery. It is not known whether the grafted cells increase production of DA or whether other processes, such as the secretion of neurotrophic factors by the marrow-derived cells, mediate the improvement in motor function (Arnhold et al., 2006; Chen et al., 2005) and neurogenesis (Chen et al., 2003; Mahmood et al., 2005). Furthermore, we cannot exclude the possibility that a small number of MSCs differentiated into dopaminergic cells in the transplanted environment as we, and others, found that MSCs express low levels of neuronal markers and the show predisposition to differentiated into mature neurons (Blondheim et al., 2006). In contrast to the MSCs, the differentiated cells demonstrated a constant

reduction in the rotational behavior until almost full recovery after 12 weeks. The improvement was associated with the survival of a considerable amount of the engrafted (GFP^+) cells. Moreover, most of the cells remained in the striatum and a significant percent of them expressed TH. Notably, the engrafted cells could be detected in the striatum-nigra track and even a few GFP-TH^+ cells integrated into the nigra. Since we have no data on the changes in the dopamine level following transplantation we cannot conclude that the engrafted cells replaced the original cells. However, we can assume that the improvement may have been due only partially to the undifferentiated MSCs and mainly to TH^+ cells. The migration of the TH^+ cells to the substantia nigra may also indicate their relevance to the relief of symptoms. Our data is similar to the reported benefits of embryonic stem cells with neuronal and dopaminergic characteristics in rodent models of PD (Ben-Hur et al., 2004; Brederlau et al., 2006; Roy et al., 2006). However, this is the first report on the symptomatic amelioration following allogeneic transplantation of TH^+-differentiated bone marrow derived cells.

In conclusion, we have shown that differentiated mouse MSCs express neuronal markers including TH and when transplanted into the striatum, can improve motor behavior in a mouse model of PD. Our findings suggest that differentiated dopaminergic neurons, generated from adult bone marrow-derived stem cells, may be used in autologous transplantation for neurorestoration in parkinsonian patients.

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