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Therapeutic Effects of Human Mesenchymal and Hematopoietic Stem Cells on Rotenone-Treated Parkinsonian Mice

Masatoshi Inden,[1] Kazuyuki Takata,[1] Kaneyasu Nishimura,[1] Yoshihisa Kitamura,[1]* Eishi Ashihara,[1] Kanji Yoshimoto,[2] Hiroyoshi Ariga,[3] Osamu Honmou,[4] and Shun Shimohama[5]

[1]Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University, Kyoto, Japan
[2]Department of Legal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
[3]Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan
[4]Department of Neural Regenerative Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
[5]Department of Neurology, School of Medicine, Sapporo Medical University, Sapporo, Japan

To appreciate the potential applications of stem cell technology in neurodegenerative diseases, including Parkinson's disease (PD), it is important to understand the characteristics of the various types of stem cells. In this study, we designed a set of experiments to compare the ability of three types of human stem cells—mesenchymal stem cells (MSCs), bone marrow $CD34^+$ cells (BM), and cord blood $CD34^+$ cells (CB)—using rotenone-treated NOD/SCID mice. Rotenone was orally administered once daily at a dose of 30 mg/kg for 56 days to induce a parkinsonian phenotype. Intravenous delivery of CB into rotenone-treated mice was slightly more beneficial than that of MSCs or BM according to both histological and behavioral analyses. Human nucleus $(hNu)^+$ cells, which are a specific marker of human cells, were observed in the striatum of rotenone-treated mice transplanted with stem cells. These hNu^+ cells expressed tyrosine hydroxylase (TH). Additionally, α-synuclein$^+$/TH^+ cells in the substantia nigra pars compacta decreased significantly following stem cell transplantation. Immunohistochemical analysis also revealed that chronic exposure to rotenone decreased glial cell line-derived neurotrophic factor immunoreactivity and that the reduction was improved by each stem cell transplantation. Gene expression analyses revealed that MSCs, BM, and CB expressed several neurotrophic factors. These results suggest that the beneficial effects of intravenous delivery of stem cells into rotenone-treated mice may result not only from a neurotrophic effect but also from endogenous brain repair mechanisms and the potential of intravenous delivery of stem cells derived from an autologous source for clinical applications in PD.

Key words: Parkinson's disease; rotenone; bone marrow; cord blood; stem cells

Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders. Its pathological characteristics include selective death of mesencephalic nigral dopamine (DA) neurons and the presence of intracytoplasmic inclusions (known as Lewy bodies) in the substantia nigra (SN), which are consistently immunostained with an antibody against α-synuclein (α-syn; Dunnett and Björklund, 1999; Shimohama et al., 2003). Although substitution of L-dihydroxyphenylalanine (L-DOPA or levodopa), a DA replacement therapy, is still considered the gold standard for patients with PD, this does not inhibit the degradation of DA neurons in the SN. In addition, motor response oscillations and drug-induced abnormal involuntary movements develop in most patients with PD who receive L-DOPA therapy for more than 5 years (Lange and Lozano, 1998; Olanow and Koller, 1998; Kitamura et al., 2003). On the other hand, an alternative approach for restoration of the damaged DA systems is transplantation of cells that synthesize DA. Allogeneic transplantation of the human fetal mesencephalon has provided proof-of-principle that cell therapy can work in patients with PD. Replacement therapy, which uses the human fetal mesencephalon transplanted into the brain, showed some symptomatic

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*Correspondence to: Yoshihisa Kitamura, PhD, Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan. E-mail: yo-kita@mb.kyoto-phu.ac.jp

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relief (Freed et al., 2001; Mendez et al., 2008). However, this strategy using the human fetal mesencephalon involves ethical issues and problems in obtaining adequate numbers of DA neurons (Dunnett and Björkland, 1999; Freed, 2002; Pawitan, 2011; Politis and Lindvall, 2012).

Stem cells have the capacity to proliferate and differentiate into multiple cellular lineages. Different classifications of stem cells reflect the range of possible cell types that they can produce and the ways in which the stem cells are derived. These stem cells include mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), embryonic stem (ES) cells, progenitor cells, and induced pluripotent stem cells (iPS; Pawitan, 2011; Politis and Lindvall, 2012). To appreciate the potential applications of stem cell technology in neurodegenerative diseases, it is important to understand the characteristics of the various available stem cell types and the potential impact of cellular therapies on disease mechanisms. Each stem cell type possesses certain qualities and advantages, and the rationale for utilizing each type depends on the desired applications and outcomes. MSCs and HSCs are an alternative source of multipotent self-renewing cells and are derived from the adult bone marrow. HSCs are also derived from umbilical cord blood. There is evidence that they can transdifferentiate to a neural lineage (Brazelton et al., 2000; Li et al., 2001; Dezawa et al., 2004; Lu et al., 2004; Sadan et al., 2009). MSCs and HSCs provide an accessible alternative to ES cells and potentially circumvent the need for immunosuppression in cellular therapies because they are derived from an autologous source.

The enormous variability in the technical procedures used in various studies has made comparison difficult. Thus, studies with different cell types under similar conditions will lead to progress in this field. In this study, we designed a set of experiments to compare the ability of three human stem cell populations—MSCs, bone marrow $CD34^+$ cells (BM), and cord blood $CD34^+$ cells (CB)—to repair PD. These cell types have become the most clinically relevant for PD cell therapies. Thus, the data presented here could be useful for designing more efficient clinical trials with fewer collateral effects.

A Details of the transplantation procedures

Group	Retenone	PBS or Injected volume	PBS or Approx. number of grafted cells	n
Rotenone	+	0.8 ml	PBS	5
MSCs	+	0.2 ml	500,000 cells	5
BM $CD34^+$	+	0.4 ml	500,000 cells	6
CB $CD34^+$	+	0.8 ml	500,000 cells	4
Control	—	0.8 ml	PBS	5

B Time course

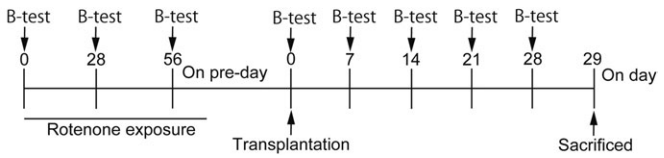

Fig. 1. Experimental design. **A:** Details of the transplantation procedures. Rotenone was orally administered to NOD/SCID mice once per day at a dose of 30 mg/kg for 56 days. The mice were divided into four subgroups (MSCs, n = 5; BM, n = 6; CB, n = 4; PBS, n = 5). CMC (0.5%) was orally administered to control mice as a vehicle (n = 5). For transplantation, MSCs, BM, or CB (approximately 500,000 cells/animal) was administered to rotenone-treated mice by injection into the tail vein on day 0. PBS was also administered to the control mice by similar injection. **B:** Time course. On days pre-0, 28, and 56, behavioral testing was performed using the rota-rod treadmill, and four subgroups were defined. Additionally, motor activity was measured on days 0, 7, 14, 21, and 28. On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. B-test, behavioral rota-rod test.

MATERIALS AND METHODS

Animals and Transplantation

NOD.CB17-*Prkdc*^{Scid}/J (NOD/SCID) mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). The mice were acclimated to and maintained at 23°C under a 12-hr light/dark cycle. All animal experiments were conducted in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals*, and the protocols were approved by the Committee for Animal Research at Kyoto Pharmaceutical University.

At the beginning of the experiment, rotenone (Sigma, St. Louis, MO) or vehicle was orally administered once per day at a dose of 30 mg/kg for 56 days. Rotenone was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC; Nacalai Tesque, Kyoto, Japan) with Tween-20. Subsequently, the suspension was orally administered once daily at a volume of 5 ml/kg body weight. CMC (0.5%) was orally administered to control mice as a vehicle. At 0, 28, and 56 days (i.e., on days pre-0, 28, and 56), motor activity was measured using the rota-rod treadmill (accelerating model 7750; Ugo Basile, Varese, Italy; Inden et al., 2011). According to the results of the rota-rod test, rotenone-treated mice were divided into four well-matched subgroups (Fig. 1A): MSCs, BM, CB, and phosphate-buffered saline (PBS) groups. Human stem cells, MSCs, BM, and CB were purchased from Takara Bio (Otsu, Japan). For transplantation, MSC, BM, or CB (approximately 500,000 cells/animal) was administered to rotenone-treated mice by injection into the tail vein on day 0 (Fig. 1). PBS was also administered to vehicle-treated (control) mice by injection into the tail vein on day 0. The rota-rod test was performed every week after the transplantation. After the last test (on day 29), the mice were sacrificed, and the brains were quickly removed for immunohistochemical analyses.

Tissue Preparation and Immunohistochemistry

On day 29 after transplantation, the mice were perfused through the aorta with 50 ml of 10 mM PBS, followed by

TABLE I. Primer Pairs Used for Reverse Transcriptase Polymerase Chain Reaction*

	Forward	Reverse
NGF	5′-gtaatgtccatgttgttctacactctg-3′	5′-ggtacaatatgagttccagtgcttt-3′
BDNF	5′- gtgtgacagtattagtgagtgggtaac-3′	5′-ggtcaatgtacatacacaagaagtgtc-3′
GDNF	5′- gacgggactttaagatgaagttatg-3′	5′-accagccttctatttctggataagt-3′
IGF-1	5′- agcatagctagagattggtgaattg-3′	5′-gtaactgcatagaagagtcagtgagtg-3′
bFGF	5′- cagttaactagggtttactgtttgagc-3′	5′-ttcaggcctaagtatactccagaatc-3′
GAPDH	5′- actctggtaaagtggatattgttgc-3′	5′-ttgtcatacttctcatggttcacac-3′

*NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

150 ml of a cold fixative consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB) under deep anesthesia with pentobarbital (100 mg/kg, i.p.). After perfusion, the brain was quickly removed and postfixed for 2 days with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4°C. The cryoprotected brain blocks were cut into 20- or 60-μm slices on a cryostat. Brain slices were incubated with primary antibodies: mouse monoclonal antibodies against tyrosine hydroxylase (TH; TH-16; diluted 1:10,000; Sigma), neuronal nuclei (NeuN; A60; 1:5,000; Chemicon, Temecula, CA), human nuclei (hNu; 235-1; 1:1,000; Millipore, Billerica, MA); and rabbit polyclonal antibody against glial cell line-derived neurotrophic factor (GDNF; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 days at 4°C. After several washes, these slices were incubated with biotinylated antibodies against mouse or rabbit IgG (1:2,000; Vector Laboratories, Burlingame, CA) as appropriate for 2 hr at room temperature. These slices were then incubated with avidin peroxidase (Vectastatin ABC Elite kit; 1:4,000; Vector Laboratories) for 1 hr at room temperature. All slices were washed several times with PBS-T between each incubation, and labeling was then visualized by 3,3′-diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) with nickel ammonium, which yielded a dark blue color.

Stereological Analysis of DA Neurons

TH^+ and also $NeuN^+$ neurons in the substantia nigra pars compacta (SNpc) were estimated in Stereo Investigator software (MBF Bioscience, Williston, VT) with stereological principles (Kitamura et al., 2010; Inden et al., 2011). Briefly, six midbrain sections (60 μm thick), each separated by a distance of 240 μm from the anterior to the posterior midbrain, were used for counting in each case. A BX51 (Olympus, Tokyo, Japan) was coupled to an Optronics Microfire digital camera CX9000 (MBF Bioscience) for visualizing the tissue sections.

Measurement of Immunoreactive Areas

The optimal density of TH^+ fiber in the striatum region was determined with a camera (Progres 3008; Carl Zeiss, Jena, Germany) and a computerized image-analysis system (WinRoof; Mitani, Fukui, Japan; Kitamura et al., 2010; Inden et al., 2011). The striatal section (20 μm thick) at 0.48 mm anterior from the bregma was selected, and the optimal density was measured within a fixed box (area = 2 × 2 mm; see Fig. 5A) positioned approximately in the middle of the striatal area. The optimal density was expressed as a percentage of the density recorded from the same area in the control mice.

Double-Immunofluorescence Staining

The brain slices were incubated with rabbit polyclonal anti-TH antibody (1:5,000; Chemicon) and mouse monoclonal anti-α-syn antibody (1:500; Transduction Laboratories, Lexington, KY) or mouse monoclonal anti-hNu (1:1,000). The primary antibodies were detected by rhodamine-labeled anti-rabbit IgG (1:500) antibody and fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody (1:500), and fluorescence was observed using a laser scanning confocal microscope (LSM510; Carl Zeiss). Semiquantitative image analysis of immunopositive cells was performed on an area of 129,600 μm^2 in the middle of SNpc.

RNA Preparation and RT-PCR

Total RNA samples from cells were prepared using an RNeasy Mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total RNA samples were reverse-transcribed by using an oligo-d(T)6 primer (Takara Bio) to prepare cDNA samples with the Omniscript RT kit (Qiagen). The cDNA samples were used as templates for RT-PCR (RT^+). For each amplification reaction, control reactions lacking RT reactions (RT^-) were performed to exclude the possibility of genomic DNA contamination. The sequence of primer sets, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), GDNF, insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is shown in Table I. A single amplified product was confirmed by electrophoresis.

Statistical Analysis

Results are expressed as mean ± SEM. The significance of differences was determined by an ANOVA and post hoc Bonferroni/Dunn test for multiple comparisons (StatView; Abacus Concepts, Berkeley, CA).

RESULTS

Rotenone-Lesioned Mice Receiving MSCs, BM, or CB Transplantation Showed Significant Improvement in Abnormal Behavior

To determine the effectiveness of the therapeutic strategies, rotenone was orally administered once daily at

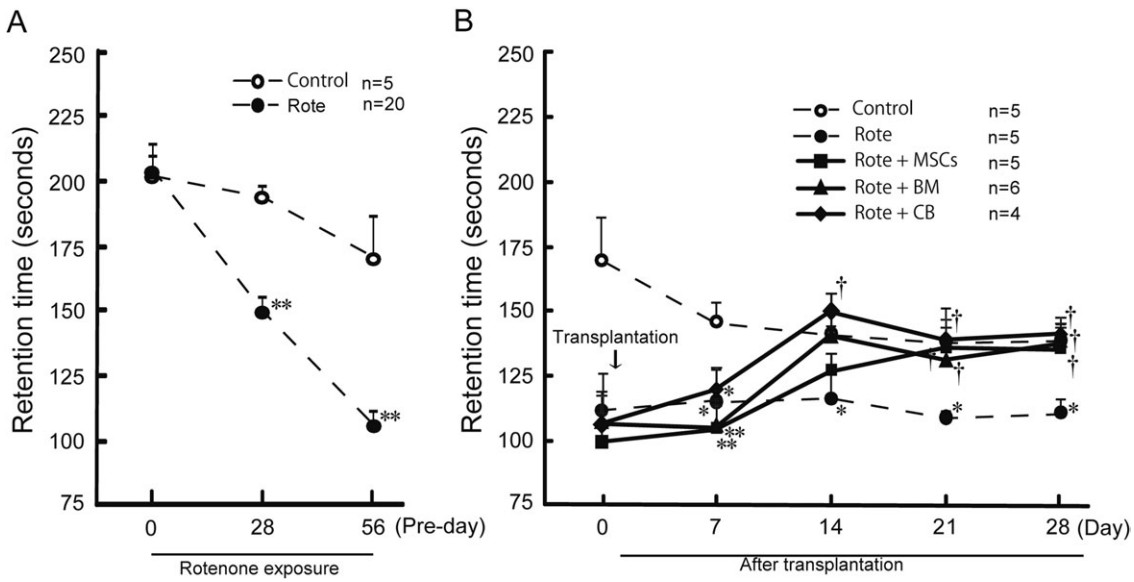

Fig. 2. Rota-rod performance in rotenone-treated mice transplanted with MSCs, BM, or CB. **A:** Rotenone induced behavioral dysfunction. Rotenone (n = 20, solid circle) or 0.5% CMC (n = 5, open circle) was orally administered once per day at a dose of 30 mg/kg for 56 days. On days pre-0, 28, and 56, motor activity was measured. The speed of the rotating rod was accelerated in a stepwise manner (10 speeds from 2 to 20 rpm for 5 min). **B:** Effect of MSCs, BM, and CB on rotenone-treated mice. For transplantation, MSCs (n = 5, solid square), BM (n = 6, solid triangle), CB (n = 4, solid diamond), or PBS (n = 5, solid circles; approximately 500,000 cells/animal) was administered to rotenone-treated mice by injection into the tail vein on day 0. PBS was also given to control mice by similar injection (n = 5, open circles). The rota-rod test was performed before (on day 0) and after (on days 7, 14, 21, and 28) transplantation. $\star P < 0.05$, $\star\star P < 0.01$ vs. control mice; $\dagger P < 0.05$ vs. rotenone-treated mice. Rote, rotenone.

a dose of 30 mg/kg for 56 days to induce a parkinsonian phenotype and then mice were transplanted with MSCs, BM, CB, or PBS through the tail vein. Before stem cell transplantation, rotenone-treated mice exhibited a marked reduction in the endurance time on the rota-rod treadmill in comparison with the control mice (rotenone-treated mice 201.4 ± 13.0, 158.9 ± 17.6, and 111.6 ± 14.7 sec; control mice 198.8 ± 4.5, 192.4 ± 5.4, and 169.8 ± 16.9 sec on days pre-0, 24, and 56, respectively; $P < 0.01$ vs. control mice; Fig. 2A). The rotenone-treated mice were divided into four subgroups (Fig. 1A), namely, MSCs, BM, CB, and PBS, and were subsequently transplanted with each type of stem cell. On day 7, there were no differences in the endurance time between the PBS-treated group and all the transplanted groups (MSCs 104.4 ± 11.2 sec; BM 104.3 ± 9.3 sec; CB 119.6 ± 8.5 sec; rotenone 115.0 ± 12.6 sec; control 146.0 ± 7.4 sec; $P < 0.05$ or $P < 0.01$ vs. control mice; Fig. 2B). On day 14, CB transplantation significantly improved behavioral dysfunction compared with that in PBS-treated rotenone-treated mice, and, although MSCs as well as BM transplantation showed an improvement trend, the improvement was insignificant (MSCs 140.2 ± 10.9 sec; BM 126.6 ± 7.1 sec; CB 149.9 ± 7.3 sec; rotenone 116.4 ± 12.4 sec; control mice 141.3 ± 3.6 sec; $P < 0.05$ vs. control mice and $P < 0.05$ vs. rotenone-treated mice; Fig. 2B). From day 21 onward, all the stem cell-transplanted groups showed a significant improvement in behavioral dysfunction (MSCs 131.4 ± 8.9 and 137.4 ± 8.2 sec; BM 135.7 ± 7.8 and 134.9 ± 8.9 sec; CB 139.1 ± 7.9 and 141.0 ± 6.6 sec; rotenone 108.9 ± 2.6 and 110.8 ± 5.6 sec; control mice 138.4 ± 13.0 and 138.8 ± 6.7 sec on days 21 and 28, respectively; $P < 0.05$ vs. control mice and $P < 0.05$ vs. rotenone-treated mice; Fig. 2B).

TH Immunohistochemical Staining in the SNpc

On day 29 after transplantation, TH staining was performed to evaluate the preserved DA neurons in the SNpc and TH^+ fibers in the striatum (Figs. 3, 5). As shown in representative photomicrographs (Fig. 3B,C), oral administration of rotenone reduced the number of TH^+ neurons in the SNpc in the rotenone-treated mice compared with that in the control mice. Stereological analysis of nigral TH^+ neurons showed that rotenone caused a significant loss of DA neurons (rotenone-treated mice 7,606 ± 1,330 cells; control mice 13,918 ± 1,338 cells; $P < 0.01$ vs. control mice; Fig. 3G). Additionally, the number of $NeuN^+$ neurons in SNpc was significantly decreased by rotenone treatment in comparison with control mice (rotenone-treated mice 60,289 ± 1,942 cells; control mice 66,227 ± 1,430 cells; $P < 0.05$ vs. control mice; Fig. 4G). MSC transplantation significantly increased the number of both TH^+ and $NeuN^+$ neurons in the SNpc compared with that in the rotenone-treated mice (TH^+ neurons 13,829 ± 1,392 cells; $NeuN^+$ neurons 66,505 ± 1,001 cells; $P < 0.05$ or $P <$

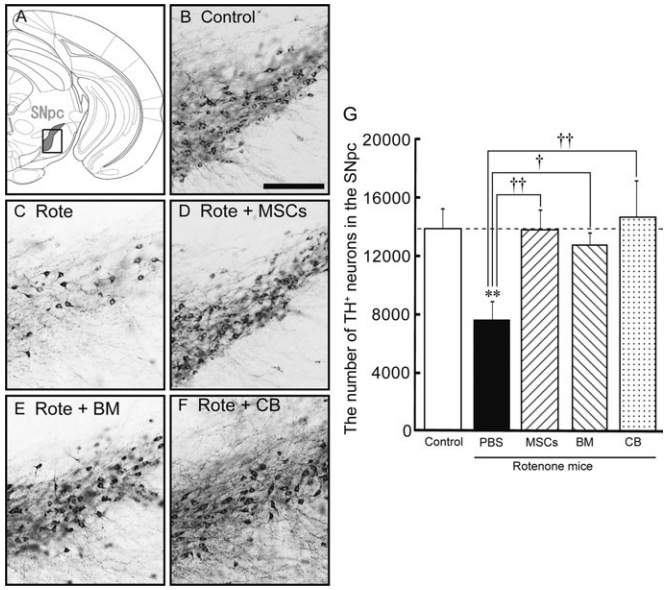

Fig. 3. Representative photomicrographs of TH^+ neurons in the SNpc. **A:** Schematic representation of the SNpc (gray area). MSCs (n = 5, **D**), BM (n = 6, **E**), CB (n = 4, **F**), or PBS (n = 5, **C**) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (n = 5, **B**). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. Midbrain slices were then immunostained with antibody against TH. **G:** Stereological analysis of TH^+ neurons in SNpc. Each value is expressed as the mean ± SEM (n = 4–6 in each group). $^{**}P < 0.01$ vs. control mice; †$P < 0.05$, ††$P < 0.01$ vs. rotenone-treated mice. Rote, rotenone. Scale bar = 200 μm.

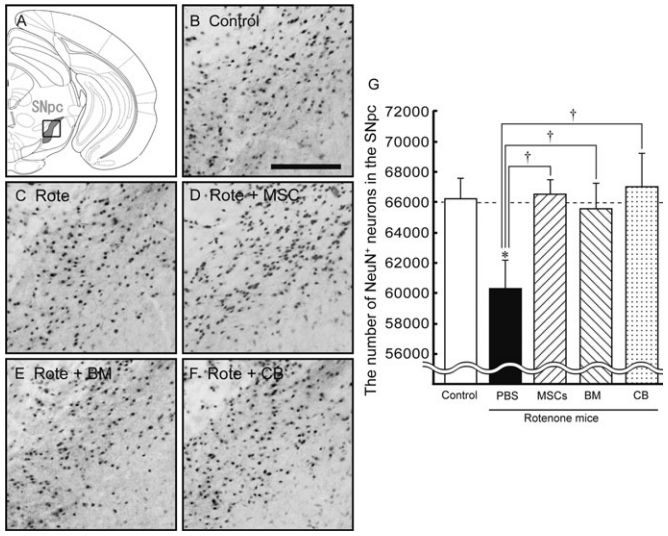

Fig. 4. Representative photomicrographs of $NeuN^+$ neurons in the SNpc. **A:** Schematic representation of the SNpc (gray area). MSCs (n = 5, **D**), BM (n = 6, **E**), CB (n = 4, **F**), or PBS (n = 5, **C**) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (n = 5, **B**). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. Midbrain slices were then immunostained with antibody against NeuN. **G:** Stereological analysis of $NeuN^+$ neurons in SNpc. Each value is expressed as the mean ± SEM (n = 4–6 in each group). $^{*}P < 0.05$ vs. control mice; †$P < 0.05$ vs. rotenone-treated mice. Rote, rotenone. Scale bar = 200 μm.

0.01 vs. rotenone-treated mice; Figs. 3C,D,G, 4C,D,G). Similarly, the number of both TH^+ and $NeuN^+$ neurons in the SNpc significantly increased with BM as well as CB transplantation (TH^+ neurons 12,775 ± 882 and 14,709 ± 2,484 cells; $NeuN^+$ neurons 65,579 ± 1,760 and 67,075 ± 2,210 cells in BM and CB transplantation, respectively; $P < 0.05$ or $P < 0.01$ vs. rotenone-treated mice, Figs. 3C,E–G, 4C,E–G).

TH Immunohistochemical Staining in the Striatum

As shown in representative photomicrographs (Fig. 5B,C), rotenone exposure evidently reduced the striatal TH^+ fiber density in the rotenone-treated mice compared with that in the control mice. Semiquantitative analysis showed that rotenone caused a significant loss of the striatal TH^+ fiber density (79.1% ± 1.2%, relative to the control mice; $P < 0.01$ vs. rotenone-treated mice; Fig. 5G). MSC, BM, or CB transplantation significantly increased the striatal TH^+ fiber density compared with that in PBS-treated rotenone-treated mice (MSCs 99.5% ± 3.9%; BM 102.1% ± 4.2%; CB 110.3% ± 7.8% relative to the control mice; $P < 0.01$ vs. rotenone-treated mice; Fig. 5D–F). On the other hand, evidence was obtained for the presence of DA neurons within the striatum (Huot et al., 2007). Although rotenone exposure reduced the striatal TH^+ neurons in the rotenone-treated mice compared with that in the control mice, MSC, BM, or CB transplantation significantly increased the striatal TH^+ neurons (MSCs 0.15 ± 0.06 cells/mm^2; BM 0.12 ± 0.03 cells/mm^2; CB 0.14 ± 0.03 cells/mm^2; rotenone-treated mice 0.02 ± 0.01 cells/mm^2; control mice 0.04 ± 0.02 cells/mm^2; $P < 0.01$ vs. rotenone-treated mice, Fig. 6A,B).

Detection of Transplanted Stem Cells

To determine whether transplanted stem cells could migrate into the lesioned nigrostriatal pathway, an immunohistochemical study was performed with the specific antibody hNu, a marker for human cells (Park et al., 2008). In the nigrostriatal pathway of both control and PBS-treated rotenone-treated mice, there were no hNu^+ cells (Fig. 6A). In contrast, a few hNu^+ cells in the striatum were observed in the rotenone-treated mice transplanted with MSCs, BM, or CB (Fig. 6A). On the other hand, in this study, hNu^+ cells were undetected in the SNpc of rotenone-treated mice transplanted with stem cells (data not shown). Additionally, other regions of brain, such as cerebral cortex and hippocampus, were analyzed, but we did not detect transplanted cells. Interestingly, hNu^+ cells were almost completely colocalized with TH^+ cells (Fig. 6A). Approximately 5.2%, 6.2%, and 5.5% of

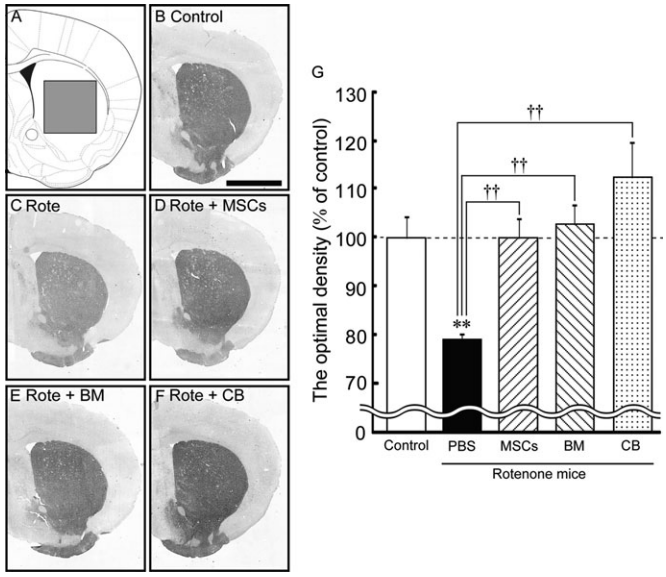

Fig. 5. Representative photomicrographs of striatal TH^+ fibers. **A:** Schematic representation of the measured area of the striatum (gray area). MSCs (n = 5, **D**), BM (n = 6, **E**), CB (n = 4, **F**), or PBS (n = 5, **C**) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (n = 5, **B**). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. Striatal slices were then immunostained with antibody against TH. **G:** Semiquantitative analysis of striatal TH^+ fibers. The optimal density was expressed as a percentage of the density recorded from the same area in the control mice. Each value is expressed as the mean ± SEM (n = 4–6 in each group). $^{**}P < 0.01$ vs. control mice; $\dagger\dagger P < 0.01$ vs. rotenone-treated mice. Rote, rotenone. Scale bar = 2 mm.

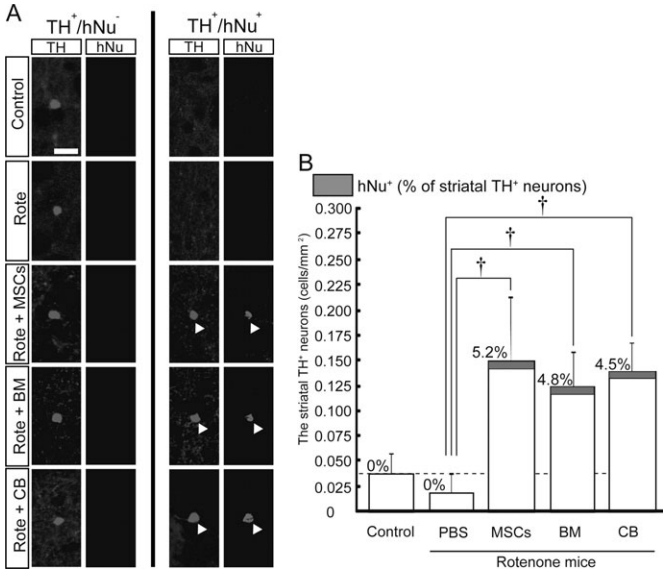

Fig. 6. Confocal microscopy analysis of hNu^+ cells in the striatum. MSCs (n = 5), BM (n = 6), CB (n = 4), or PBS (n = 5) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (n = 5). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. **A:** Striatal slices were immunostained with antibodies against TH and hNu and then analyzed by laser scanning confocal microscopy. Arrowheads indicate TH^+ and hNu^+ neurons. **B:** Semiquantitative analysis of striatal TH^+ neurons (cells/mm^2) and hNu^+ cells (% of striatal TH^+ neurons; gray area). Each value is expressed as the mean ± SEM (n = 4–6 in each group). $\dagger P < 0.05$ vs. rotenone-treated mice. Rote, rotenone. Scale bar = 20 μm.

striatal TH^+ neurons were colocalized with hNu immunoreactivity in the rotenone-treated mice transplanted with MSCs, BM, and CB, respectively (Fig. 6B).

Effect of Transplanted Stem Cells on Intracellular α-Syn and GDNF Immunoreactivity in the SNpc

Previous studies have shown that oral administration of rotenone induced a high level of cytoplasmic α-syn immunoreactivity in some surviving TH^+ neurons in the SNpc (Inden et al., 2007, 2011). Confocal microscopic analysis was performed to examine the effect of transplanted stem cells on intracellular α-syn immunoreactivity in the SNpc. Although TH^+ neurons were clearly detected in the SNpc of the control mice, α-syn immunoreactivity was not detected in these TH^+ neurons (Fig. 7A). Exposure of rotenone increased α-syn immunoreactivity (Fig. 7A). Approximately 29.2% of surviving TH^+ neurons were colocalized with α-syn in the SNpc of the rotenone-treated mice ($P < 0.01$ vs. control mice; Fig. 7B). $\alpha\text{-Syn}^+$ cells significantly decreased with each stem cell transplantation (Fig. 7A). Approximately 5.4%, 4.8%, and 4.5% of surviving TH^+ neurons were colocalized with α-syn in the SNpc of the rotenone-treated mice transplanted with MSCs, BM, and CB, respectively ($P < 0.01$ or $P < 0.001$ vs. rotenone-treated mice; Fig. 7B).

We also performed immunohistochemical analysis using an anti-GDNF antibody. Immunohistochemial analysis revealed that chronic exposure to rotenone decreased GDNF immunoreactivity in the SNpc on day 29 (Fig. 8A,B), and the reduction was improved by each stem cells transplantation (Fig. 8C,E)

Expression of Trophic Factor mRNAs in MSCs, BM, or CB

Previous studies have shown that stem cells produce several neurotrophic factors (Shintani et al., 2007; Hokari et al., 2008; Zheng et al., 2010). The expression of mRNA species coding neurotrophic factors was examined in each of the stem cells. Gene expression analysis using RT-PCR demonstrated that MSCs, BM, and also CB expressed NGF, BDNF, IGF-1, and bFGF. On the other hand, GDNF was detected only in MSCs (Fig. 9).

DISCUSSION

To understand the pathophysiology of PD and develop therapies to improve symptom management, it is

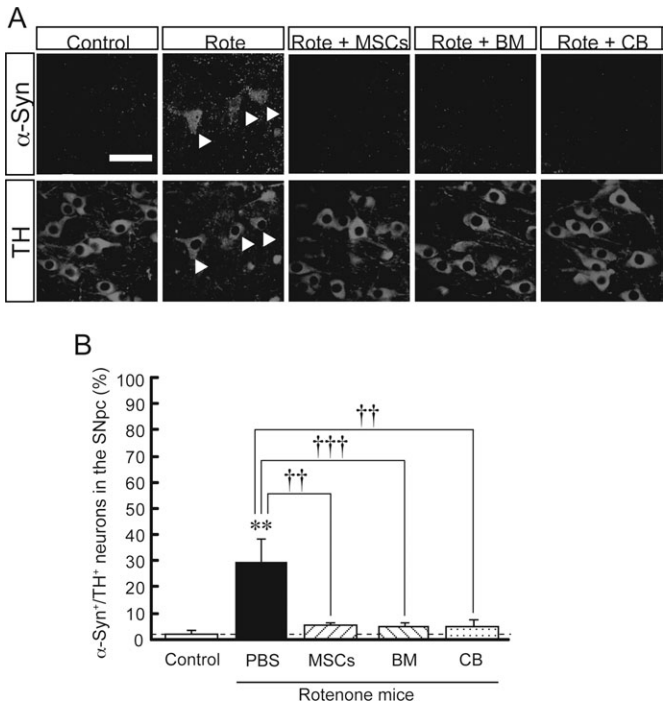

Fig. 7. Intracellular expression of α-syn in TH^+ neurons of the SNpc. MSCs (n = 5), BM (n = 6), CB (n = 4), or PBS (n = 5) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (n = 5). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. **A:** Midbrain slices were immunostained with antibodies against TH and α-syn and then analyzed by laser scanning confocal microscopy. Arrowheads indicate TH^+ and $\alpha\text{-syn}^+$ neurons. **B:** Semiquantitative analysis of $\alpha\text{-syn}^+/TH^+$ neurons in the SNpc. Analysis of immunopositive cells was performed on an area of 129,600 μm^2 in the middle of the SNpc. Each value is expressed as the mean ± SEM based on the number of TH^+ neurons (n = 4–6 in each group). $^{**}P < 0.01$ vs. control mice; $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. rotenone-treated mice. Rote, rotenone. Scale bar = 50 μm.

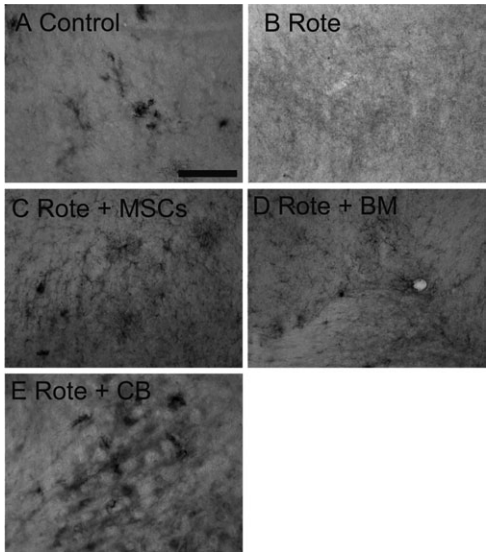

Fig. 8. Representative photomicrographs of GDNF immunoreactivity in the SNpc. **A–E:** MSCs (C), BM (D), CB (E), or PBS (B) was administered to rotenone-treated mice by injection into the tail vein. PBS was also administered to control mice by similar injection (A). On day 29, the mice were sacrificed, and their brains were quickly removed for immunohistochemical analysis. Midbrain slices were then immunostained with an antibody against GDNF. Rote, rotenone. Scale bar = 30 μm.

important to have relevant disease models of PD in which new pharmacological agents and treatment strategies can be assessed before initiating clinical trials (Shimohama et al., 2003). Although the available neurotoxin models of PD are not perfect, we used rotenone-treated mice as an animal model of PD in this study. We have reported that chronic oral administration of rotenone caused specific nigrostriatal DA neurodegeneration in C57BL/6 mice (Inden et al., 2007, 2011). In rotenone-treated mice, α-syn immunoreactivity increased in some surviving TH^+ neurons in the SNpc. The previous experimental condition in the rotenone model may resemble early PD symptoms rather than atypical parkinsonism, although long-term treatment with rotenone is necessary (Inden et al., 2007, 2011). In this study, we used NOD/SCID mice, which permitted human cell engraftment (Uchida et al., 2000; Cummings et al., 2005). Chronic oral administration of rotenone caused nigrostriatal DA neurodegeneration as well as behavioral impairment in NOD/SCID mice similar to that in previous reports using C57BL/6 mice (Inden et al., 2007, 2011). In addition, $\alpha\text{-syn}^+/TH^+$ neurons in the SNpc were increased by chronic administration of rotenone in the rotenone-treated mice compared with that in the control mice. Therefore, we investigated the effect of peripheral administration of stem cells such as MSCs, BM, and also CB on the nigrostriatal DA system under chronic oral administration of rotenone using NOD/SCID mice as an in vivo model of PD.

In this study, intravenous delivery of CB into rotenone-treated mice was relatively more efficient than that of MSCs or BM, and this was confirmed in both histological and behavioral analyses. MSCs and BM were harvested from bone marrow at the adult stage. Both the differentiation and the proliferation potential of MSCs and BM decrease significantly with age. On the other hand, CB occupy an intermediate-age stage between the embryonic stem cells and adults stem cells. Therefore, CB have a higher differentiation capability and proliferate more rapidly in response to cytokines stimulation than do their counterparts in other stem cells (Hao et al., 1995; Martins et al., 2009; Ali and Bahbahani, 2010). The advantage of CB potential could be responsible for the differences in neuroprotective effects. Although the mechanism responsible for the therapeutic effects of intravenous delivery of MSCs, BM, or CB on rotenone-treated mice remains unclear, there are several possibilities. One possibility is that these cells may

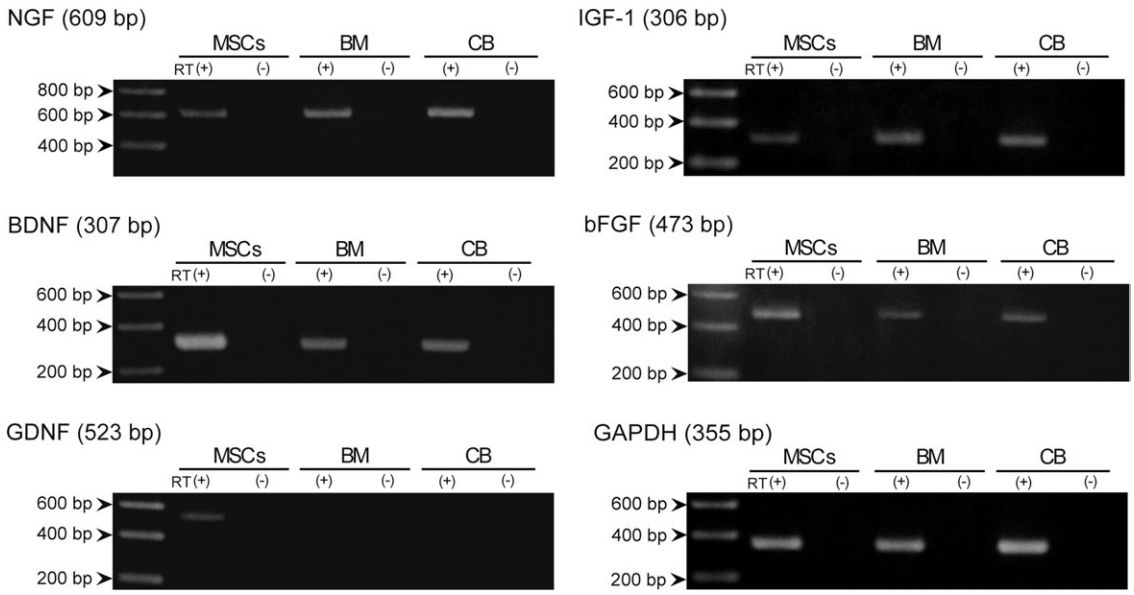

Fig. 9. Demonstration of mRNA expression of neurotrophic factors in MSCs, BM, and CB by RT-PCR. mRNA expression of NGF (609 bp), BDNF (307 bp), GDNF (523 bp), IGF-1 (306 bp), bFGF (473 bp), and GAPDH (355 bp). The cDNA samples were used as templates for RT-PCR (RT^+). For each amplification reaction, control reactions lacking RT reactions (RT^-) were performed to exclude the possibility of genomic DNA contamination. The sequence of primer sets is shown in Table I.

provide a neuroprotective function. Previous studies showed that stem cells released neurotrophic factors (Dezawa et al., 2004; Q. Chen et al., 2005; Shintani et al., 2007; Hokari et al., 2008, 2009; Fierro et al., 2011; Bassi et al., 2012). Previous studies also showed a significant increase in the ischemic or 6-OHDA-lesioned striatum of the stem cells-transplanted group compared with vehicle-treated control group (Shyu et al., 2006, 2009). Several trophic factors have a protective effect on DA neurons in vitro and in vivo (Date, 1996; Shintani et al., 2007; Peterson and Nutt, 2008). Briefly, GDNF, BDNF, NGF, IGF-1, and bFGF have been reported to act on DA neurons in vitro and in vivo, making them potential therapeutic catalysts for PD. For instance, in animal models of PD, intraventricular injection of GDNF induces a long-term increase in the striatal DA content (Hoffer et al., 1994), although clinical trials using intraventricular injections of GDNF did not improve functional deficits in patients with PD and nigrostriatal function was not augmented (Kordower et al., 1999). Thus, intraventricular GDNF in humans appears to be the wrong method (Kordwer, 2003). Cell therapy is an alternative technique for providing neurotrophic factors, including GDNF, to DA neurons of the nigrostriatal pathway (Fernandez-Espejo, 2004). In this study, we aimed to demonstrate a potential neuroprotective method to prevent cell death or even stimulate regeneration in the damaged nigrostriatal DA system by using stem cells that expressed neurotrophic factors. In our study, a few hNu^+ cells in the striatum were observed in rotenone-treated mice by immunofluorescence staining. Nevertheless, intravenous delivery of stem cells exerted effective therapeutic effects. Immunohistochemial analysis using an anti-GDNF antibody also revealed that chronic exposure to rotenone decreased GDNF immunoreactivity in the SNpc on day 29, and the reduction was improved by each stem cell transplantation (Fig. 8), indicating that the brain environment indeed improved as a result of transplanted stem cells. These results suggest that secreted neurotrophic factors from stem cells such as MSCs, BM, and CB may play a key role in the neuroprotective effects.

Another possibility is that the transplanted stem cells may integrate into and replace the DA neurons injured by chronic exposure of rotenone. MSCs, BM, or CB can differentiate into neural phenotypes in vitro (Brazelton et al., 2000; Li et al., 2001; Dezawa et al., 2004; Lu et al., 2004; Sadan et al., 2009). Cell fusion has recently been found to occur when bone marrow stromal cells (BMSC) are transplanted into various types of organs, including the brain (Hokari et al., 2008). However, we detected a few hNu^+ cells in the striatum, similar to the numbers seen in previous studies (Shen et al., 2007; Wang et al., 2010). We have reported that chronic oral administration of rotenone caused specific nigrostriatal DA neurodegeneration in C57BL/6 mice (Inden et al., 2007). On the other hand, Betarbet et al. (2000) demonstrated that in rats, especially Lewis rats, chronic systemic exposure to rotenone through jugular vein cannulation reproduced many features of PD,

including nigrostriatal DA neurodegeneration, and suggested that striatal nerve terminals were affected earlier and more severely by rotenone than nigral cell bodies. In addition, anatomically, the area of striatum is much larger than that of SN. Therefore, although the mechanisms remain unclear, many stem cells may migrate into a more severely lesioned site. Thus, in this study, we detected transplanted cells in the striatum, but not the SN, on day 29 after transplantation.

On the other hand, in this study, MSCs, BM, or CB transplantation was seen to increase the number of TH^+ as well as $NeuN^+$ neurons in the SNpc. Multipotent neural stem cells are harbored in the adult mammalian brain (Goldman, 2005; Borta and Höglinger, 2007), suggesting the potential for self-repair of the mature brain. A previous study demonstrated that the SN of adult rats contains progenitor cells with the potential of differentiating into glial cells and neurons (Lie et al., 2002). Previous studies have also demonstrated that 6-OHDA lesioning in rats or MPTP lesioning in mice results in cell proliferation in the SN without apparent DAergic differentiation (Kay and Blum, 2000; Lie et al., 2002; Steiner et al., 2006; Aponso et al., 2010). Furthermore, another study has indicated that DAergic differentiation occurred at a very low level in the SN of normal mice and occurred at an increased level after MPTP lesioning (Zhao et al., 2003). In addition, basal levels of neurogenesis, increased proliferation, and DAergic differentiation following MPTP have been demonstrated in nestin-LacZ transgenic mice (Shan et al., 2006). The effect of growth factors on DAergic neurogenesis in the adult SN has also been investigated. The number of nigral TH^+ neurons increased by 32% after chronic infusion of GDNF into the striatum of adult rats, which was most likely the result of induction of a DAergic phenotype in pre-existing neurons, although no evidence for the generation of bromodeoxyuridine $(BrdU)^+/TH^+$ neurons was found (Y. Chen et al., 2005). However, the concept of cell differentiation and neurogenesis in the midbrain region still remains a controversial topic, because conflicting findings have been obtained in previous studies (Kay and Blum, 2000; Mao et al., 2001; Lie et al., 2002; Cooper and Isacson, 2004; Frielingsdorf et al., 2004; Y. Chen et al., 2005; Mohapel et al., 2005; Reimers et al., 2006; Steiner et al., 2006). In this study, the number of TH^+ and $NeuN^+$ neurons in the SNpc of the rotenone-treated mice with transplanted stem cells was significantly increased compared with that in PBS-treated rotenone-treated mice. Additionally, MSCs, BM, and also CB expressed several neurotrophic factors. Thus, we assume that transplantation of MSCs, BM, or CB may promote endogenous brain repair mechanisms, although we found no obvious evidence that progenitor cells in the SN can differentiate into DA neurons in the in vivo condition. Further studies are needed to resolve the detailed mechanism of endogenous brain repair by stem cell transplantation.

Previous studies have shown that chronic exposure of rotenone produced some TH^+ neurons, which induced a high level of cytoplasmic α-syn immunoreactivity in the SNpc (Inden et al., 2007, 2011). Genetic studies led to the discovery of a small percentage of familial PD cases linked directly to genetic mutations, as well as gene duplications and triplications. The first gene associated with PD was α-syn (*PARK1*; Polymeropoulos et al., 1997). Furthermore, duplications and triplications of α-syn are linked to an early-onset familial PD (*PARK4*; Singleton et al., 2003). These genetic studies suggest that an excess increase of α-syn protein levels may represent a gain of toxic function. In this study, $\alpha\text{-syn}^+/TH^+$ cells in the SNpc decreased on stem cell injection into the tail vein. Although the exact mechanism remains unclear, neuroprotective effects of stem cells could involve a reduction in intracellular α-syn, and the results of this study indicated that stem cell transplantation may be a useful therapy for patients with PD as well as for those with other α-synucleinopathies such as multiple system atrophy and dementia with Lewy bodies.

In conclusion, the present study provides evidence that stem cell (MSCs, BM, or CB) transplantation exerts neuroprotection against nigrostriatal DA neurodegeneration by chronic exposure to rotenone. We have demonstrated that the beneficial effects of intravenous delivery of stem cells into rotenone-treated mice may result not only from a neurotrophic effect but also from endogenous brain repair mechanisms. These results demonstrate the potential of intravenous delivery of stem cells derived from an autologous source for clinical applications in PD, although further studies are required.

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