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Research Article

Intrastriatal grafts of mesenchymal stem cells in adult intact rats can elevate tyrosine hydroxylase expression and dopamine levels

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Abstract

MSCs (mesenchymal stem cells) derived from the bone marrow have shown to be a promising source of stem cells in a therapeutic strategy of neurodegenerative disorder. Also, MSCs can enhance the TH (tyrosine hydroxylase) expression and DA (dopamine) content in catecholaminergic cells by *in vitro* co-culture system. In the present study, we investigated the effect of intrastriatal grafts of MSCs on TH protein and gene levels and DA content in adult intact rats. When MSCs were transplanted into the striatum of normal rats, the grafted striatum not only had significantly higher TH protein and mRNA levels, but also significantly higher DA content than the untransplanted striatum. Meanwhile, the grafted MSCs differentiated into neurons, astrocytes and oligodendrocytes; however, TH-positive cells could not be detected in our study. These experimental results offer further evidence that MSCs are a promising candidate for treating neurodegenerative diseases such as Parkinson's disease.

Keywords: dopamine; mesenchymal stem cells; transplantation; tyrosine hydroxylase

1. Introduction

PD (Parkinson's disease) is clinically characterized by the development of bradykinesia, rigidity and a resting tremor, which has been attributed in part to the selective loss of dopaminergic neurons in the substantia nigra pars compacta and the consequent loss of DA (dopamine) in the striatum. Drug therapies including levodopa and DA agonists and neurosurgical interventions such as deep brain stimulation can be used to effectively treat many of the symptoms of PD. However, these therapies cannot stop the disease from progression (The Deep-Brain Stimulation for Parkinson's Disease Study Group, 2001; Krack et al., 2003; Isacson, 2004; Chapuis et al., 2005).

Cell-based therapies have been developed as an alternative therapeutic strategy. Although embryonic stem cells and fetal ventral mesencephalon are a reliable source for the generation of neural cells for grafting in PD, their use has raised ethical concerns. Among the various stem cells, BM (bone marrow)-derived MSCs (mesenchymal stem cells) have been shown as a promising source of stem cells in cell therapy. MSCs are also clinically attractive because they are easily harvested and autologously transplanted in humans and their use is not limited by ethical issues. Jiang et al. (2002) have proven that MSCs are pluripotent cells with the potential to differentiate into a variety of cells with mesoderm, ectoderm and endoderm characteristics. Numerous studies suggest that MSCs have the capacity to differentiate into neural lineages *in vitro* (Brazelton et al., 2000; Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Jiang et al., 2002). Several groups have also proven that MSCs are capable of differentiating into neural cells *in vivo* (Kopen et al., 1999; Muñoz-Elias et al., 2004; Liu et al., 2009). In addition, we have shown that MSCs enhance the TH (tyrosine hydroxylase) protein levels in PC12 cells and ventral mesencephalic cells by *in vitro* co-culture system respectively (Jin et al., 2007, 2008). In the present study, we investigated whether MSCs can elevate TH expression and DA levels in the striata of adult intact rats.

2. Materials and methods

2.1. Isolation and culture of rat MSCs

Animal experiments were carried out 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 Gyeongsang National University. After BM aspiration (bilateral femurs and tibias) from male Sprague–Dawley rats aged 8 weeks, mononuclear cells were separated via Ficoll® 400 (1.077 g/ml; Sigma) density gradient centrifugation. These cells were cultured in Dulbecco's modified

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Abbreviations: BM, bone marrow; DA, dopamine; DPBS, Dulbecco's phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; MOSP, myelin/oligodendrocyte-specific protein; MSC, mesenchymal stem cell; PD, Parkinson's disease; RT-PCR, reverse transcription-PCR; TH, tyrosine hydroxylase.

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Eagle's high-glucose medium (4.5 g/l glucose; Gibco-BRL) containing 10% FBS (fetal bovine serum; Gibco-BRL) and penicillin (100 units/ml)/streptomycin (100 mg/ml) (Sigma) at 37℃ with 5% CO_2 in air in a humidified incubator for 48 h. Non-adherent cells were removed by changing the medium and the resulting monolayer of cells (the BM-derived MSCs) was trypsinized. Aliquots were cultured further or frozen and stored. The MSCs were used for the following experiments when they were at the fourth passage. Identification of mesenchymal cells has previously been reported by flow cytometric determination (Jin et al., 2008).

2.2. Transplantation of rat MSCs

To assess the effect of rat MSC transplantation on TH protein, TH mRNA and DA levels in the normal adult rat striatum, 48 male Sprague–Dawley rats (200–250 g) were divided into four groups: (1) an intact normal group ($n=16$); (2) a sham-operated group that only received a scalp incision ($n=16$); (3) a vehicle group injected with DPBS (Dulbecco's phosphate-buffered saline; Gibco) containing 0.2% BSA (Sigma) ($n=16$); and (4) a group transplanted with MSCs ($n=16$). MSCs were suspended in DPBS containing 0.2% BSA. Recipient rats were anaesthetized with Ketalar [7 mg/100 g, i.p. (intraperitoneally)] and Rompun (1 mg/100 g, i.p.) and mounted in a stereotaxic frame. Using a 10-μl Hamilton syringe (22-gauge), a single deposit of a 5-μl cell suspension (8×10^4/μl) was injected over a 5-min period into the right striatum at the following co-ordinates: A-P=+0.5 mm, M-L=3.0 mm and D-V=−5.5 mm. After the transplantation, the needle of the Hamilton syringe was left in place for an additional 5 min and then slowly withdrawn. A piece of bone wax was applied on to the skull defect to prevent the solution from leaking. Vehicle rats received injections of 5 μl of DPBS at the same co-ordinates. In addition, to evaluate differentiation of MSCs *in vivo*, MSCs were prelabelled before transplantation using Dil-Ac-LDL (1:20; Biomedical Technologies) as per established protocols (Cheng et al., 2008).

The rats were anaesthetized with lethal doses of sodium pentobarbital 4 weeks after implantation of MSCs into their right striatum. The brains were removed and treated for corresponding detection respectively.

2.3. Western blot analysis to determine TH protein levels

Right striata from each group ($n=4$) were lysed in ice-cold SDS-sample lysis buffer containing 10 mM Tris/HCl (pH 8.0), 5 mM EDTA, 0.15 M NaCl and 10% Triton X-100, and centrifuged (12000 rev./min, 4℃, 10 min), after which the supernatant was collected. The total protein (30 μg) of each sample was separated by SDS/PAGE and transferred electrophoretically on to nitrocellulose filters. Non-specific antibody binding was blocked by incubation with 5% non-fat dry milk for 1 h at room temperature (25℃). Immunoblotting was performed by using antibodies against TH (1:500, polyclonal, Chemicon) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:300, monoclonal; Chemicon). The membrane was then washed and incubated with horseradish peroxidase–conjugated anti-rabbit IgG (1:1000; Santa Cruz Biotechnology) or anti-mouse IgG (1:5000; Chemicon). The blots were developed using the enhanced chemiluminescence method (Amersham Biosciences).

2.4. Semi-quantitative RT-PCR (reverse transcription-PCR)

Total RNA of the right striata from each group ($n=4$) were extracted by using TRIzol™ (Gibco-BRL) and 2 μg of total sample RNA was reverse-transcribed with oligo(dt)15 and random hexamer primers by using AMV reverse transcriptase (Boehringer Mannheim). A total of 2 μl of cDNA was used with 2.5 units of Taq DNA polymerase (Promega) for PCR amplification in a thermal cycler under the following conditions: one cycle at 94℃ (5 min); 30 cycles of 94℃ (1 min), 57℃ (1 min) and 72℃ (2 min); one final extension cycle of 72℃ (10 min) for TH; one cycle at 95℃ (5 min); 30 cycles of 95℃ (30 s), 59℃ (30 s) and 72℃ (1 min); a final extension cycle of 72℃ (10 min) for GAPDH. The PCR products were run on a 1% agarose gel containing ethidium bromide and viewed under UV light. The PCR band intensities for TH mRNA were expressed as ratios relative to the GAPDH band intensities. For each gene, the PCR was run three times. The 5′ to 3′ primer sequences for TH and GAPDH are as follows: TH: F, 5′-ATGCCCACCCCCAGCGCCCC-3′; R, 5′-GACACTTTTCTTGGGAACCA-3′ (513 bp). GAPDH: F, 5′-CTCGTGGAGTCTACTGGTGT-3′; R, 5′-GTCATCATACTTGGCAGGTT-3′ (420 bp).

2.5. Measurement of DA levels by HPLC

For neurochemical analysis, the right striata from each group ($n=4$) were weighed, homogenized in 200 μl of 0.1 M perchloric acid and centrifuged (15000 ***g***) for 15 min at 4℃. The DA concentrations in the samples were determined against an external standard by HPLC coupled to a variable UV-Spectro Monitor 3200 detector. The column was packed with polymethacrylate gel (6 mm × 150 mm ID, Shodex® DE-613). The mobile phase consisted of 0.05 M potassium dihydrogen phosphate and 0.05% phosphoric acid, pH 3.0, with a flow rate of 0.6 ml/min. The signal was detected at 200 nm.

2.6. Immunohistochemical analysis

To confirm if the differentiation potential of MSCs *in vivo* were involved in the enhanced effect of MSCs on TH expression in striatum, rats from each group ($n=4$) were anaesthetized with sodium pentobarbital (50 mg/kg) and perfusion-fixed with 4% buffered paraformaldehyde after a normal saline flush. Brains were removed and post-fixed in the same fixative for 24 h, followed by transfer to 20% (w/v) sucrose in PBS overnight at 4℃, embedded in OCT compound and frozen in liquid nitrogen. Serial coronal sections were cut through the striatum at 15 μm thickness in a cryostat.

To qualitatively identify neural differentiation of MSCs *in vivo*, anti-β III-tubulin (1:400, mouse monoclonal; Sigma), anti-GFAP (glial fibrillary acidic protein; 1:400, rabbit polyclonal; Chemicon), anti-MOST (myelin/oligodendrocyte-specific protein, 1:1000 dilution, mouse monoclonal; Chemicon) and anti-TH (1:500, rabbit

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polyclonal; Chemicon) were used as primary antibodies, and FITC-conjugated anti-mouse IgG (1:64; Sigma) and anti-rabbit IgG (1:80; Sigma) were used as the secondary antibodies. The sections were treated with 10% normal goat serum for 30 min to block the non-specific staining, and then incubated with the primary antibodies at room temperature for 2 h, followed by incubation with the secondary antibodies at 37°C for 30 min. Nuclei of the cells were counterstained using Hoechst 33342 (5 μg/ml; Sigma) for 5 min at room temperature. The slides were examined with a fluorescent microscope (Olympus, Japan).

2.7. Statistical analysis

Results are expressed as means ± S.D. Statistical analysis was conducted using one-way ANOVA, followed by a post-hoc LSD (least significant difference) test. A value of $P<0.05$ was considered statistically significant. The SPSS software package (SPSS, version 13.0) was used for all statistical tests.

3. Results

3.1. TH protein level

Western blot analysis was performed to determine whether the grafted MSCs affected TH protein expression in transplanted ipsilateral striata. Representative immunoblots are shown in Figure 1(A). Groups of intact, sham and vehicle-injected rats served as controls. Relative to the GAPDH signal, the 60-kDa TH protein signal was statistically stronger in the MSC group compared with control groups (Figure 1B). There was no significant difference observed among the intact group, sham group and vehicle group.

3.2. TH mRNA expression

RT-PCR was used to determine whether TH mRNA expression of ipsilateral striata was affected by transplanted MSCs. A representative image is shown in Figure 2(A). Relative to the GAPDH signal, the MSC group expressed significantly higher levels of TH mRNA than control groups (Figure 2B). There was no significant difference observed among the normal group, sham group and vehicle group.

3.3. DA level

A group of four rats were injected in the right striatum with 4×10^5 MSCs. Four weeks later, the right striata were analysed for DA levels by HPLC analysis. Representative HPLC traces are shown in Figures 3(A)–3(D). The right striatum DA levels in the MSC group (20005 ± 1173 ng/mg of wet tissue weight) were significantly higher compared with those in control groups (intact

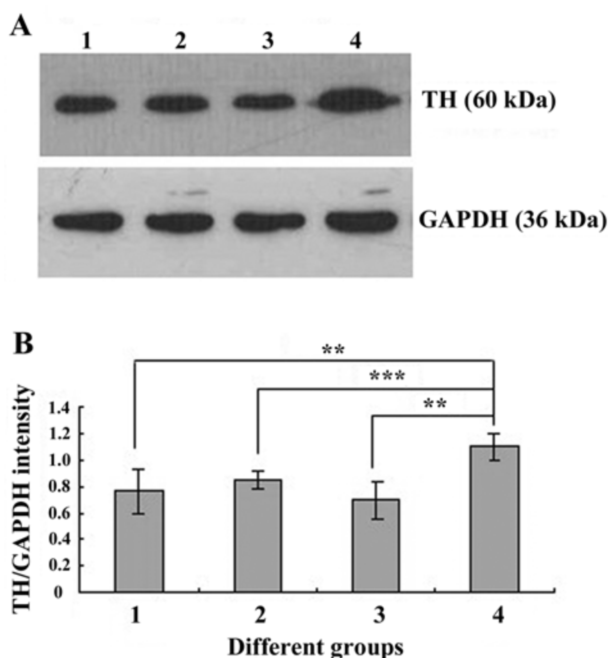

Figure 1 Effect of MSC transplantation on expression of TH protein in the rat striatum
(**A**) Representative Western blot of TH protein. Rats were injected in the right striatum with 4×10^5 MSCs (MSC group, 4) or vehicle (PBS group, 3), or were sham-operated (sham group, 2) or intact (normal group, 1). Four weeks later, TH protein in the right striata was assayed by immunoblotting analysis. (**B**) Ratio of TH to GAPDH density value was determined by densitometric analysis. Values shown are means ± S.D. TH protein levels for each group (n=4). (**$P<0.01$; ***$P<0.001$).

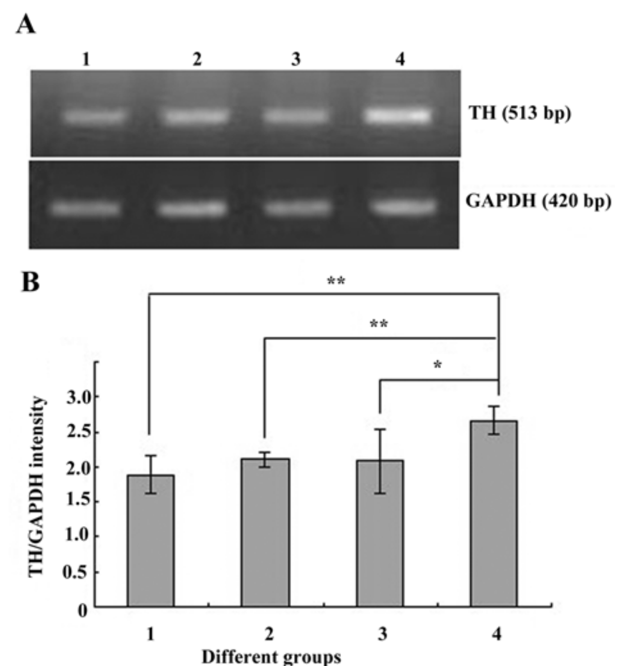

Figure 2 Effect of MSC transplantation on expression of TH mRNA in the rat striatum
(**A**) Representative RT-PCR of TH mRNA. Rats were injected in the right striatum with 4×10^5 MSCs (MSC group, 4) or vehicle (PBS group, 3), or were sham-operated (sham group, 2) or intact (normal group, 1). Four weeks later, TH mRNA detection in the right striata was performed by RT-PCR. (**B**) Ratio of TH to GAPDH band intensities was determined by densitometric analysis. Values shown are means ± S.D. TH mRNA levels for each group (n=4). (*$P<0.05$; **$P<0.01$).

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group, 17878 ± 511; sham group, 16392 ± 1302; vehicle group, 15984 ± 2383 ng/mg of wet tissue weight) (Figure 3E). There was no significant difference observed among the normal group, sham group and vehicle group.

3.4. Immunohistochemistry of neural differentiation

By immunohistochemistry, numerous dil pre-labelled cells injected stereotaxically remained confined to the injected site 4 weeks after transplantation, whereas a small number of cells

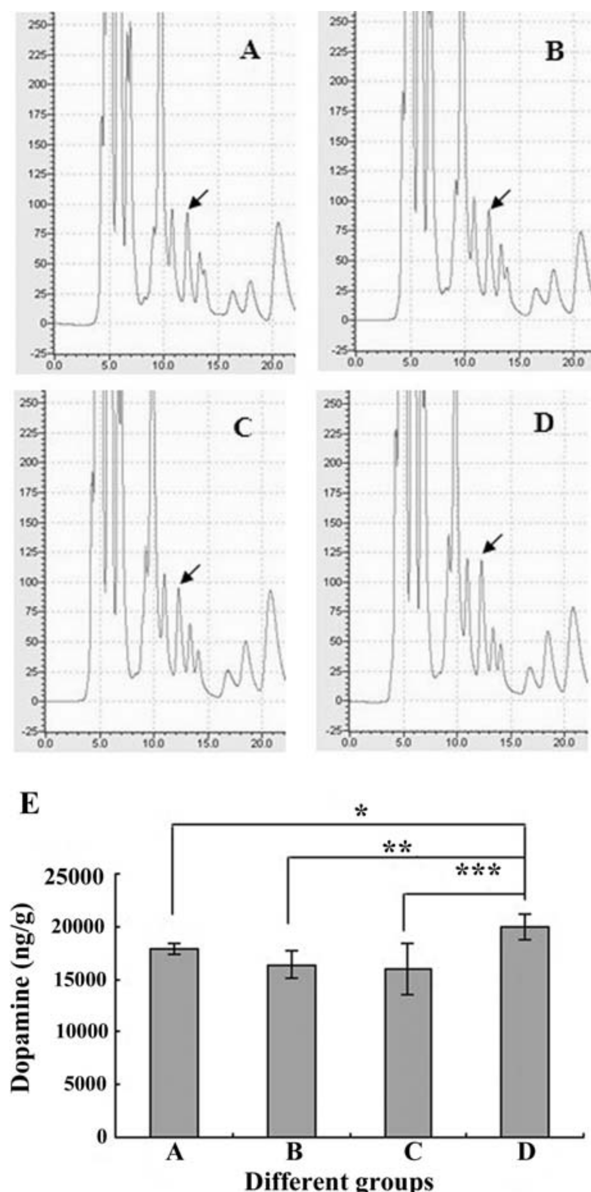

Figure 3 Effect of MSC transplantation on DA levels in the rat striatum
Rats were injected in the right striatum with 4×10^5 MSCs (MSC group, **D**) or vehicle (PBS group, **C**), or were sham-operated (sham group, **B**) or intact (normal group, **A**). Four weeks later, the DA level in the right striata was assessed by HPLC. The HPLC pattern for each group, as determined by using a UV detector. Arrows indicate the DA peaks. (**E**) Values indicate the means ± S.D. DA levels for each group ($n=4$). (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

were observed at a short distance migration. MSCs gave rise to cells expressing neuronal, astrocyte and oligodendrocyte markers. Some transplanted MSCs were immunopositive for neuronal marker β III-tubulin (Figures 4B and 4C), astrocyte marker GFAP (Figures 4E and 4F) and oligodendrocyte marker MOSP (Figures 4H and 4I) (Dyer et al., 1991). However, MSCs were immunonegative for TH protein, indicating that the MSCs had not differentiated into dopaminergic cells (Liu et al., 2009; Zwart et al., 2009). These cells were mainly found at the site of transplantation. Although transplanted MSCs exhibited immunophenotypes of neuronal cells, the external morphology of these cells at 4 weeks after grafting appeared to be rounded in outline with very few projection fibres.

4. Discussion

In the present study, we examined whether transplantation of intact rat striata with rat MSCs up-regulated their expression of TH protein and mRNA and DA levels. Consistent with our previous studies (Jin et al., 2007, 2008), we found that the MSC-transplanted rats had significantly higher TH expression and DA levels compared with untransplanted ones. Meanwhile, the grafted MSCs differentiated into neurons, astrocytes and oligodendrocytes; however, TH-positive cells could not be detected in our study.

MSCs can not be only derived from BM, but can also be isolated from other sources such as adipose tissue, umbilical cord blood and mobilized peripheral blood. They all have been used in transplant studies (Zuk et al., 2001; Willing et al., 2003; Koh et al., 2008). Although MSCs are pluripotent cells with the potential for neural cell differentiation, many researchers have found a small number of cells expressing markers of neural lineage after transplantation (Li et al., 2001; Cízková et al., 2006). Vallières and Sawchenko (2003) have observed that BM cells expressing green fluorescent protein do not even show neuronal differentiation in *in vivo* trials. In addition, Li et al. (2001) have shown that only 0.8% of cells transplanted into a mouse PD model differentiate into TH-positive cells. In our study, although we have not detected dopaminergic differentiation of grafted MSCs, they have shown glial cell markers (Figure 4). This is consistent with the results reported by Kopen et al. (1999). Interestingly, MSCs (Garcia et al., 2004; Mahmood et al., 2004; Crigler et al., 2006) and astrocytes (Schaar et al., 1993; Moretto et al., 1994; Müller et al., 1995) express a variety of neuroregulatory molecules, including NGF (nerve growth factor), BDNF (brain-derived nerve growth factor) and GDNF (glial cell-derived neurotrophic factor). Therefore, other mechanisms for functional recovery have been suggested to account for the action of transplanted MSCs, such as an elevated level of neurotrophic factors in the injured animal brain (Li et al., 2001; McCoy et al., 2008). Additionally, MSCs can enhance the TH expression of PC12 cells and the DA content of fetal ventral mesencephalon *in vitro* (Jin et al., 2007, 2008). Therefore, enhanced TH expression and DA levels in ipsilateral striata may be, at least in part, attributed to the neurotrophic support of MSCs and/or differentiated glial cells (Li et al., 2007; Arien-Zakay et al., 2009; Zwart et al., 2009).

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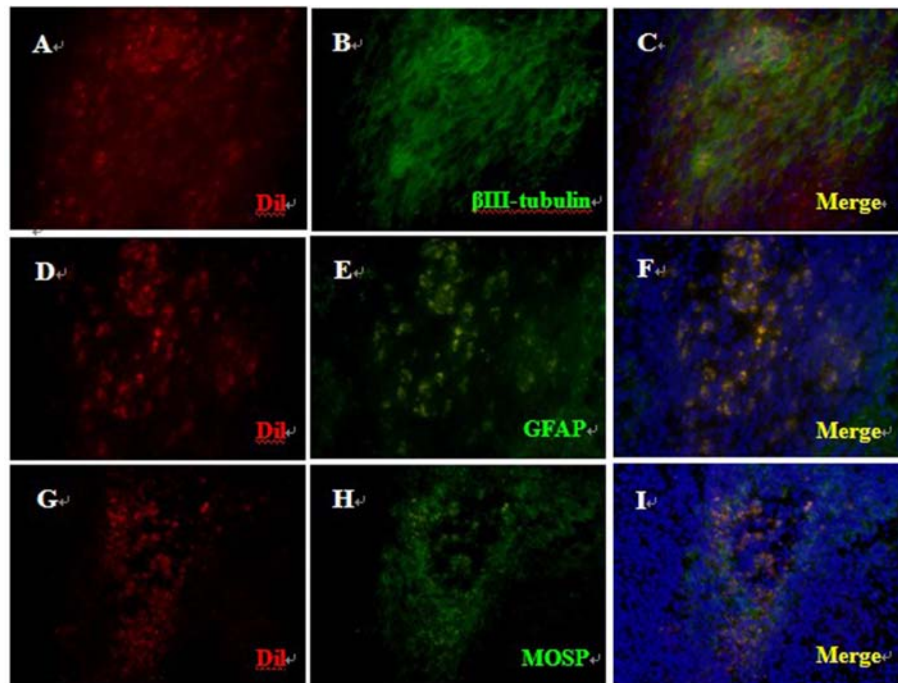

Figure 4 Fluorescence micrographs of a graft 4 weeks after implantation of rat BM-derived MSCs into the right intact adult rat striatum
(**A**, **D** and **G**) MSCs prelabelled with Dil-Ac-LDL (red). MSCs (green) stained with an anti-β III-tubulin antibody (**B**), an anti-GFAP antibody (**E**) and an anti-MOSP antibody (**H**). (**C**, **F** and **I**) Merged images. Nuclei of the cells were counterstained with Hoechst 33342. All images are shown at an original magnification of $\times$200.

The results presented in this study indicate that the *in vivo* transplantation of MSCs in rat striata not only up-regulate their expression of TH protein and mRNA, but also increase their DA levels. Further studies are warranted to understand the exact mechanism of the reasonable application of MSC transplantation.

Author contribution

Guang-Zhen Jin was responsible for the design, analysis and writing of the manuscript; Su-Jin Cho and Young-S Lee took charge of data collection and analysis; Myeong-Ok Kim and Dong-Woo Cho undertook an analysis of the study; Il-Keun Kong contributed to the design and analysis of the study and acknowledges all individuals who have contributed to this work.

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