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Bone marrow-derived mesenchymal stem cells increase dopamine synthesis in the injured striatum☆

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Abstract
Previous studies showed that tyrosine hydroxylase or neurturin gene-modified cells transplanted into rats with Parkinson's disease significantly improved behavior and increased striatal dopamine content. In the present study, we transplanted tyrosine hydroxylase and neurturin gene-modified bone marrow-derived mesenchymal stem cells into the damaged striatum of Parkinson's disease model rats. Several weeks after cell transplantation, in addition to an improvement of motor function, tyrosine hydroxylase and neurturin proteins were up-regulated in the injured striatum, and importantly, levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid increased significantly. Furthermore, the density of the D2 dopamine receptor in the postsynaptic membranes of dopaminergic neurons was decreased. These results indicate that transplantation of tyrosine hydroxylase and neurturin gene-modified bone marrow-derived mesenchymal stem cells increases dopamine synthesis and significantly improves the behavior of rats with Parkinson's disease.

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Key Words
Parkinson's disease; tyrosine hydroxylase; neurturin; bone marrow-derived mesenchymal stem cells; transplantation; dopamine; gene therapy; neurodegenerative disease; regeneration; neural

Research Highlights
(1) After transplantation of tyrosine hydroxylase and neurturin gene-modified bone marrow-derived mesenchymal stem cells into the damaged corpus striatum of Parkinson's disease model rats, the levels of tyrosine hydroxylase, neurturin and dopamine increased in the striatum, while the density of the D2 dopamine receptor in the postsynaptic region of dopaminergic synapses decreased.
(2) Transplantation of tyrosine hydroxylase and neurturin gene-modified bone marrow mesenchymal stem cells into the corpus striatum significantly improved motor function in Parkinson's disease model rats.

Abbreviations
PD, Parkinson's disease; DA, dopamine; BMSCs, bone marrow-derived mesenchymal stem cells; TH, tyrosine hydroxylase; NTN, Neurturin

INTRODUCTION

Parkinson's disease (PD) is a progressive hypokinetic neurological disorder that is characterized by abnormal posture, bradykinesia, rigidity, akinesia and resting tremor[1]. The major pathological finding in PD is the loss of nigrostriatal dopaminergic neurons, with a resultant decrease in dopamine (DA) and the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC)[2]. Currently, the main treatments for PD include pharmacological treatments, surgery, cell transplantation and gene therapy[3-5].

Recent progress in stem cell biology provides hope for neural repair using gene-modified bone marrow-derived mesenchymal stem cells (BMSCs)[6-7]. Autologous BMSC transplantation should prevent immune rejection and overcome ethical problems associated with gene therapy[8-9]. BMSCs are easy to cultivate and proliferate rapidly *in vitro*, and have thus become ideal carriers for modified genes[10].

Tyrosine hydroxylase (TH) is a major dopamine synthesizing enzyme and is the signature protein of DA neurons within the neural system[11-12]. Augmentation of TH in the striatum can increase the level of DA in the brain and alleviate apomorphine-induced rotation, which is beneficial in the treatment of PD[13].

Neurturin (NTN) is a growth factor that is structurally and functionally related to glial-derived neurotrophic factor[14]. It belongs to the glial-derived neurotrophic factor family and is characterized by its capacity to nourish, support, protect and repair damage to dopaminergic neurons[15]. NTN promotes the survival of embryonic midbrain neurons *in vitro* and *in vivo*. It protects mature dopaminergic neurons in different PD models, and prevents neurodegeneration[16-18]. Overall, it appears that NTN is an interesting candidate for the treatment of PD.

Previous studies found that TH- or NTN-modified cells transplanted into rats can improve PD-related behavior and restore and increase DA content[19-20]. Further studies showed that single-gene transfection was not sufficient to maintain dopamine levels or continuously improve symptoms. Recent studies suggest that co-transfection of multiple genes is required for effective treatment[21-22]. However, it is unknown whether combined transfection of TH and NTN could be therapeutic. The present study investigated the effect of co-transfection of these two genes on cells.

RESULTS

Biological identification of rat BMSCs *in vitro*

Sub-cultured BMSCs were evaluated for cell number using the MTT method. Absorbance (*A*) value measurements indicated that the number of BMSCs increased with the length of cultivation (Figure 1A). Passage 3 BMSCs were analyzed using flow cytometry. The positive rate for CD29 was 93.93%, and that for CD34 was only 2.40% (Figure 1B). The expression of CD29 and CD34 was also assessed using immunohistochemistry. CD29 labeling was observed as yellow staining in the cytoplasm of BMSCs. However, no CD34 was observed (Figure 1C).

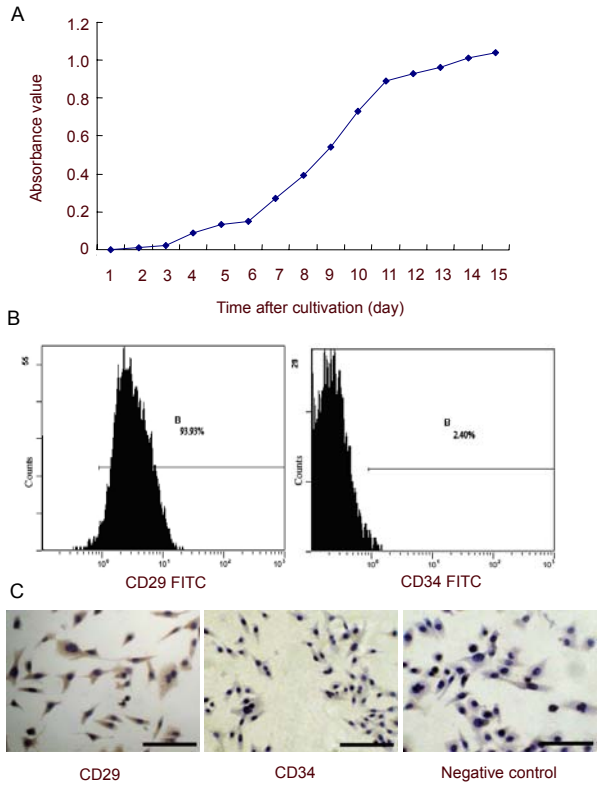

Figure 1 Biological identification of rat bone marrow-derived mesenchymal stem cells (BMSCs) *in vitro*.

(A) Absorbance value increases with duration of BMSC cultivation.

The expression of CD29 and CD34 in passage 3 BMSCs was analyzed by flow cytometry (B) and immunohistochemistry (C). (B) The positive rate of CD29 was 93.93%, and for CD34, it was only 2.40%. (C) Positive expression of CD29 in the cytoplasm and negative expression of CD34 were confirmed by immunohistochemistry.

Arrows indicate positive expression of CD29 and CD34. Scale bars: 100 μm.

Co-expression of TH and NTN in TH-NTN-BMSCs

Real-time PCR showed that TH and NTN expression in

TH-NTN-BMSCs was significantly higher than in BMSCs ($P < 0.05$). NTN and TH protein expression in transfected TH-NTN-BMSC cell lysates was measured with enzyme-linked immunosorbent assay at 24, 36, 48 and 72 hours. Expression gradually increased with time, demonstrating a high transfection efficiency (Figure 2).

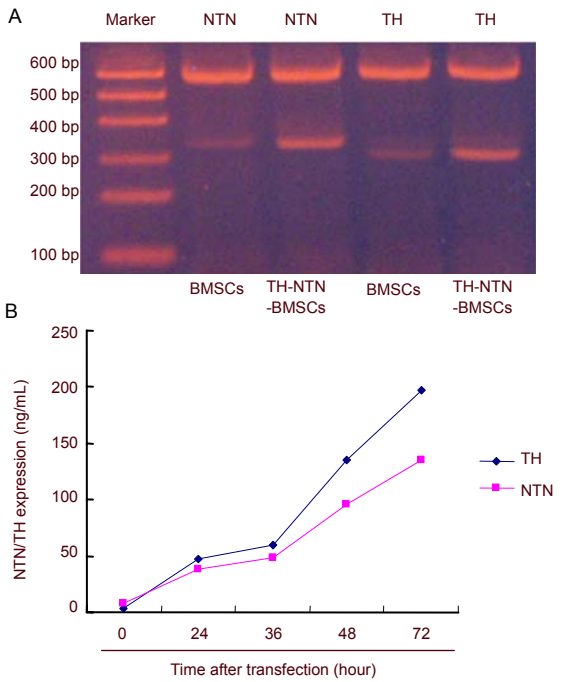

Figure 2 Co-expression of TH and NTN in transfected TH-NTN-BMSCs.

(A) Real-time PCR revealed that levels of TH and NTN transcripts in TH-NTN-BMSCs was higher than that in BMSCs. TH: 284 bp; NTN: 332 bp.

(B) NTN and TH protein expression in transfected TH-NTN-BMSC cell lysates was measured by enzyme-linked immunosorbent assay. The increase in expression in stably transfected cell lines indicates the efficiency of transfection.

TH: Tyrosine hydroxylase; NTN: neurturin.

Quantitative analysis of experimental animals

A total of 190 Sprague-Dawley rats were used, and 20 were randomly selected for bone marrow harvesting under sterile conditions. Ten rats from the remaining 170 were randomly selected as a control group, and 160 rats were used to model PD by injecting 6-hydroxydopamine (6-OHDA) into the corpus striatum. Two weeks after the 6-OHDA injection, the 114 surviving PD model rats were peritoneally injected with apomorphine (0.5 mg/kg) to assess behavior, and 84 successful PD model rats were confirmed. The 84 model rats were equally and randomly assigned to three groups: PD, BMSC and TH-NTN-BMSC groups. Rats in the BMSC and TH-NTN-BMSC groups were separately transplanted with BMSCs and TH-NTN-BMSCs, respectively, into the right striatum at the same time.

TH-NTN-BMSCs improved motor function in PD model rats

Two weeks after cell transplantation, the number of rotations decreased significantly in the BMSC and TH-NTN-BMSC groups compared with the PD group ($P < 0.05$). At 4, 6 and 8 weeks after cell transplantation, there was no difference between PD and BMSC groups. However, in the TH-NTN-BMSC group, the number of rotations was significantly lower than in the PD and BMSC groups ($P < 0.05$; Figure 3).

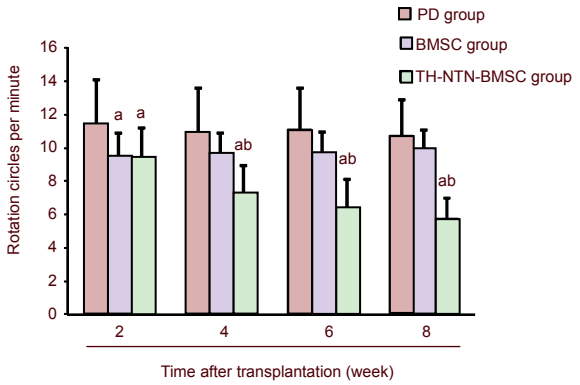

Figure 3 Rotation frequencies after apomorphine peritoneal injection following cell transplantation.

Data are presented as mean ± SD, and statistical significance was determined using one-way analysis of variance. $^{a}P < 0.05$, *vs*. PD group; $^{b}P < 0.05$, *vs*. BMSC group.

PD: Parkinson's disease; TH: tyrosine hydroxylase; NTN: neurturin; BMSCs: bone marrow-derived mesenchymal stem cells.

Immunohistochemical confirmation of successful transplantation in the right striatum of PD model rats

Immunohistochemistry for 5-bromodeoxyuridine was performed in the right striatum samples. In the BMSC group, 5-bromodeoxyuridine-labeled BMSCs were clearly visible 2 weeks after transplantation, but disappeared at 8 weeks. In the TH-NTN-BMSC group, labeled TH-NTN-BMSCs were found from 2 to 8 weeks (Figure 4).

TH-NTN-BMSC transplantation enhanced expression of TH and NTN in the right striatum of PD model rats

Eight weeks after cell transplantation, TH and NTN labeling was examined in the PD, BMSC and TH-NTN-BMSC groups. In the BMSC group, TH and NTN expression was similar to that in the PD group ($P > 0.05$); however, the expression of these factors was significantly increased in the TH-NTN-BMSC group compared with the other groups ($P < 0.05$; Figures 5A, B).

Western blotting gave similar results (Figure 5C).

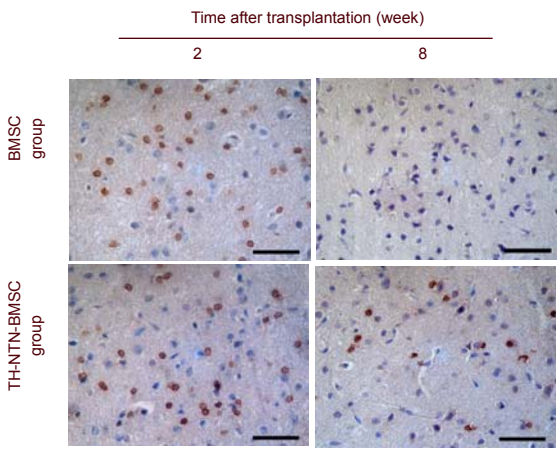

Figure 4 Immunohistochemistry for transplanted cells in the right striatum of PD model rats at 2 and 8 weeks after transplantation.

After 2 weeks, both BMSCs and TH-NTN-BMSCs were observed in the right corpus striatum. After 8 weeks, only TH-NTN-BMSCs were still present in the striatum. Red arrowheads show 5-bromodeoxyuridine-labeled cells. Scale bars: 100 μm.

PD: Parkinson's disease; TH: tyrosine hydroxylase; NTN: neurturin; BMSCs: bone marrow-derived mesenchymal stem cells.

TH-NTN-BMSC transplantation increased DA and DOPAC levels in the right striatum in PD model rats

The concentrations of DA and DOPAC were measured using a high pressure liquid chromatography/electrochemical detection system to evaluate the difference between the left and right striatum in PD model rats. The levels of DA and DOPAC in the left striatum were significantly higher than in the 6-OHDA injection side (right side; $P < 0.05$; Figure 6A). The results show that 6-OHDA damages dopamine neurons, resulting in a decrease in the levels of DA and its metabolite, DOPAC. Differences between the PD, BMSC and TH-NTN-BMSC groups were also found at different time points after cell transplantation (Figures 6B, C). In the TH-NTN-BMSC group, DA and DOPAC levels steadily increased over time from 2 to 8 weeks after cell transplantation ($P < 0.05$).

Dopamine receptor density in dopaminergic synapses in the striatum

To investigate D1 and D2 receptor density in dopaminergic synapses, immunogold post-embedding on ultrathin sections from the corpus striatum was performed. D1 receptors were found situated on the postsynaptic membranes (black arrowheads). D2 receptors appeared mostly on the postsynaptic membranes and were seldom situated on the presynaptic membranes (Figure 7A). The density of D1 receptors in the postsynaptic membrane region was similar among the control, PD, BMSC and TH-NTN-BMSC groups ($P > 0.05$). However, the D2 receptor densities in the PD, BMSC and TH-NTN-BMSC groups were significantly higher than in the control group ($P < 0.05$). Furthermore, a lower density of D2 receptors was found in the TH-NTN-BMSC group than in the BMSC group ($P < 0.05$; Figure 7B).

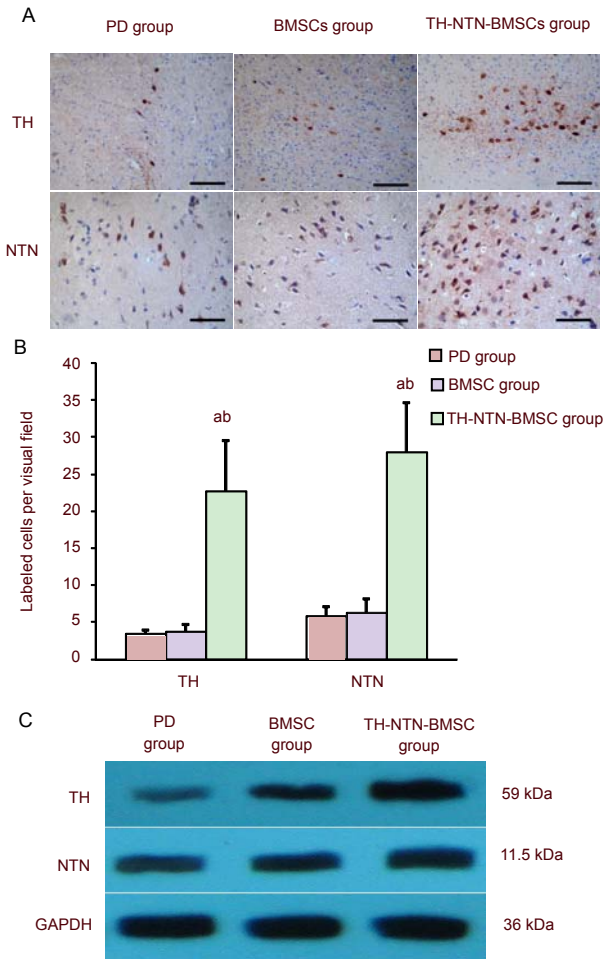

Figure 5 TH and NTN detection in the right corpus striatum at 8 weeks after cell transplantation.

(A) Both TH and NTN labeling (red arrowheads) were much higher in the TH-NTN-BMSC group compared with the PD and BMSC groups (immunohistochemistry). Scale bars: 100 μm.

(B) Data are expressed as mean ± SD, and statistical significance was determined using one-way analysis of variance. $^{a}P < 0.05$, *vs*. PD group; $^{b}P < 0.05$, *vs*. BMSC group.

(C) Western blot analysis of extracts from right corpus striatum brain tissue demonstrated stronger expression of both TH and NTN proteins in the TH-NTN-BMSC group.

PD: Parkinson's disease; TH: tyrosine hydroxylase; NTN: neurturin; BMSCs: bone marrow-derived mesenchymal stem cells; GADPH: glyceraldehyde-3-phosphate dehydrogenase.

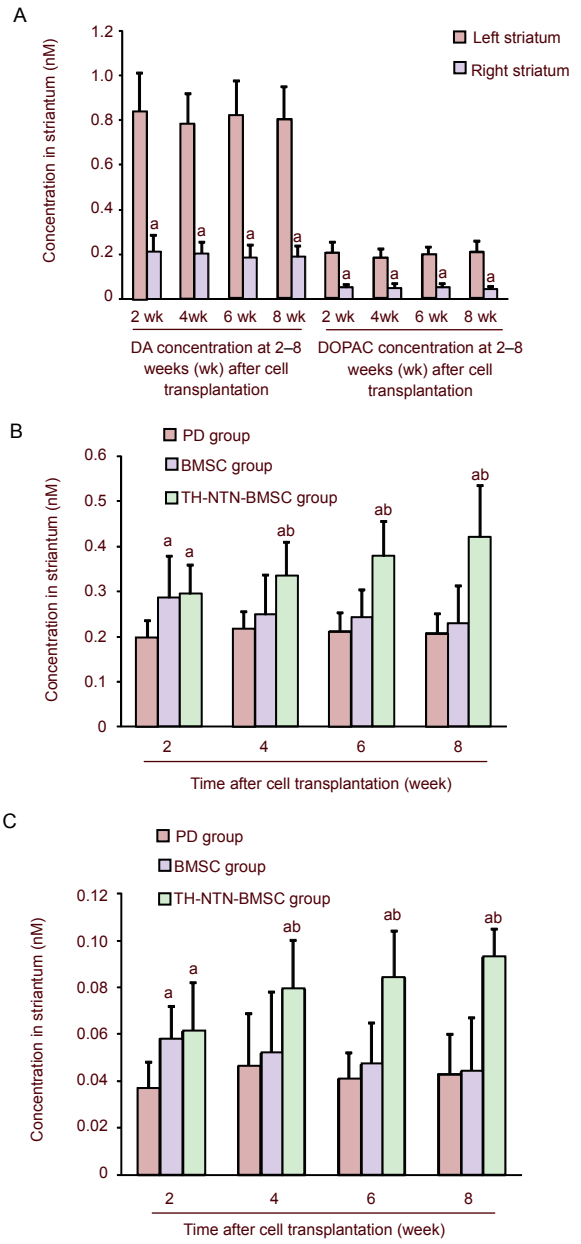

Figure 6 Influence of TH-NTN-BMSC transplantation on DA and DOPAC content in right striatum of PD rats.

Data are presented as mean ± SD, and statistical significance was determined using one-way analysis of variance.

(A) In the PD group, the DA and DOPAC levels in the right striatum were much lower than in the left side. $^{a}P < 0.05$, *vs*. left striatum.

The DA (B) and DOPAC (C) concentration in the right striatum after cell transplantation. Both DA and DOPAC were significantly increased in the TH-NTN-BMSC group compared with the PD and BMSC groups, except for at 2 weeks after cell transplantation. $^{a}P < 0.05$, *vs*. PD group; $^{b}P < 0.05$, *vs*. BMSC group.

PD: Parkinson's disease; TH: tyrosine hydroxylase; NTN: neurturin; BMSCs, bone marrow-derived mesenchymal stem cells; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid.

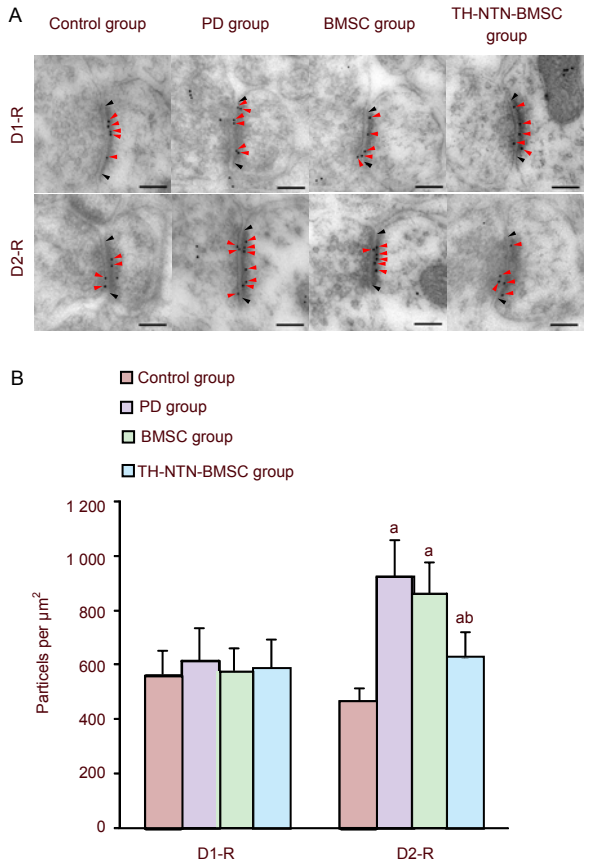

Figure 7 Dopamine receptor density in the dopaminergic synapses of the corpus striatum in PD model rats.

(A) Electron micrographs from the corpus striatum in all groups with D1 and D2 receptors. D1 receptors (D1-R, red arrowheads) were found situated on the postsynaptic membranes (black arrowheads). D2 receptors (D2-R red arrowheads) appeared mostly on the postsynaptic membranes (black arrowheads) and were seldom situated on the presynaptic membranes. Scale bars: 100 nm.

(B) D1-R and D2-R densities in the postsynaptic membranes. No intergroup differences were found for D1-R densities. The D2-R densities were significantly lower in the control group than in all other groups. The density of D2-R was lower in the TH-NTN-BMSC group than in the BMSC group. Data are presented as mean ± SD, and statistical significance was determined using one-way analysis of variance. $^{a}P < 0.05$, *vs*. control group; $^{b}P < 0.05$, *vs*. BMSC group.

PD: Parkinson's disease; TH: tyrosine hydroxylase; NTN: neurturin; BMSCs: bone marrow-derived mesenchymal stem cells.

DISCUSSION

A stable and reliable PD model is essential for PD research. Although PD does not develop spontaneously in animals, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 6-OHDA are accepted as inducers of PD in

rodents[23-25]. Because of the lack of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine receptors in rats, unilateral 6-OHDA injection of the corpus striatum is used to model PD in rats. 6-OHDA in the substantia nigra or corpus striatum can be taken up by dopamine neurons through dopamine transporters and competitively inhibits dopamine. This leads to dysfunction of mitochondrial respiratory complex I and dopamine neuron apoptosis[26]. Peritoneal injection of the dopamine receptor agonist apomorphine may lead to the observed contralateral (uninjured side) asymmetric rotation[27]. The occurrence and frequency of rotation is considered a measure of modeling success and can also be used to evaluate the degree of injury and the efficacy of therapy[28]. In this study, 2 weeks after modified cell transplantation, rotation frequency decreased significantly in the BMSC and TH-NTN-BMSC groups. This demonstrates that both BMSCs and TH-NTN-BMSCs may alleviate PD symptoms within a short period after transplantation. However, the results from 4, 6 and 8 weeks showed that only the TH-NTN-BMSC group exhibited decreased rotation frequency. BMSC transplantation alone was not sufficient to treat dopamine neuron damage; the expression of TH and NTN by these cells was required for therapeutic efficacy.

Immunohistochemistry results from the BMSC and TH-NTN-BMSC groups 2 and 8 weeks after transplantation may help explain the observed behavioral effect. In the BMSC group, 5-bromodeoxyuridine-labeled BMSCs were clearly observed 2 weeks after transplantation, but disappeared at 8 weeks. In the TH-NTN-BMSC group, the labeled TH-NTN-BMSC cell density was almost the same at 2 and 8 weeks. Thus, it appears that BMSCs cannot survive in the corpus striatum for 8 weeks, while TH-NTN-BMSCs can endure for a longer period. The enhanced persistence of these cells likely underlies the improvement in behavior.

Wang's research showed that the transplantation of astrocytes expressing both TH and brain-derived neurotrophic factor in PD model rats produced a good therapeutic effect[13]. To investigate the effect of TH and NTN in the corpus striatum, we performed immunohistochemistry and western blotting 8 weeks after cell transplantation. Our results in the TH-NTN-BMSC group support the high efficacy of TH and NTN combination therapy. However, the effect of increasing TH and NTN protein levels remained to be addressed. Thus, DA and DOPAC were measured using the high performance liquid electrochemical measurement system. DA and DOPAC levels were significantly higher in the TH-NTN-BMSC group 8 weeks after cell transplantation. Our results revealed that the TH and NTN genes were transcribed, translated and expressed in the transplanted TH-NTN-BMSCs. The increase in NTN protein levels likely protects dopamine neurons by inhibiting their apoptosis. Elevated TH levels can also increase the amount of DA and DOPAC in the corpus striatum. The combined protective effects of TH and NTN improved motor function in PD rats.

However, the observed difference in protein expression is not sufficient to explain the effects of TH-NTN-BMSC transplantation in PD rats[29]. D1 and D2 receptor density in dopaminergic synapses were quantified by immunogold post-embedding in ultrathin sections from the corpus striatum. At 8 weeks after cell transplantation, D1 receptor expression was similar in the control, PD, BMSC and TH-NTN-BMSC groups. However, the D2 receptor densities in the PD, BMSC and TH-NTN-BMSC groups were significantly higher than in the control group. A lower density of D2 receptors was also found in the TH-NTN-BMSC group compared with the BMSC group. The up-regulation of D2 receptors could result from increased numbers of D2 receptors on the remaining dopaminergic neuron terminals or increased D2 receptor synthesis within striatopallidal neurons[30-31]. After treatment with TH-NTN gene-modified cells, the elevated D2 receptor levels returned to normal, although D1 receptor levels remained unaltered.

The PD model generated by 6-OHDA unilateral striatal injection is useful for PD research. After TH-NTN-BMSC transplantation into the injured striatum of PD rats, behavior was significantly improved; transplanted cells survived after 8 weeks, and TH and NTN proteins from gene-modified cells were produced. Furthermore, levels of DA and its metabolite DOPAC increased significantly concomitant with the change in D2 receptors on the dopaminergic neuron terminals.

In conclusion, TH and NTN expression by BMSCs increases DA synthesis and also protects dopaminergic neurons in the lesioned striatum, providing dual therapeutic effects. Our results may represent a significant advancement for PD gene therapy.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

The study was performed at the Henan Provincial People's Hospital, China from May 2010 to June 2012.

Materials

One hundred and ninety male Sprague-Dawley rats, 2–3 months old, 180–200 g, were provided by the Experimental Animal Center of Henan Province (animal license: No.4104035). Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[32].

Methods

In vitro culture and identification of rat BMSCs

A total of 20 rats were ether-anesthetized and decapitated. Bone marrow was obtained from rats under sterile conditions. BMSCs were separated *in vitro* by density gradient centrifugation for primary culture at 37°C in air containing 5% CO_2[33]. Sub-cultured BMSCs were monitored using the MTT method[34] to guarantee appropriate growth. Passage 3 BMSCs were analyzed by flow cytometry and immunohistochemistry for the expression of CD29 and CD34, and prepared for transformation with the pIRES-TH-NTN vector *in vitro* [35-36].

Construction and expression of pIRES-TH-NTN vector in vitro

The TH and NTN gene sequences were cloned from pMD18T-TH and pcDNA-NTN (TaKaRa Bio, Dalian, China), respectively. The constructed pIRES-TH-NTN vectors were obtained from the Molecular lab in Zhengzhou University. Liposome 2000 (Invitrogen, Chicago, IL, USA) was used to transfect BMSC lines with pIRES-TH-NTN, and the transfected cells were selected under G418 (Inalco, Paris, France). The co-expression of TH and NTN mRNA in TH-NTN-BMSCs was assessed with real-time PCR[37]. Primers used for amplification of the 284-bp TH were 5'-TCT GGA ACG GTA CTG TGG CT-3' and 3'-CAA TGT CCT GGG AGA ACT GG-5'. Primers used for amplification of the 332-bp NTN were 5'-CCC TGC TGT CTG TCT GGA TGT G-3' and 3'-ACG GTT TCG TCC GAC GTG TAG-5'. NTN and TH protein expression in cell lysates was measured by enzyme-linked immunosorbent assay[38] at 24, 36, 48 and 72 hours to evaluate transfection efficiency. After G418 selection, stably transfected TH-NTN-BMSCs were prepared for transplantation.

Establishment of PD models

A PD model was established by two-point injection of 10 μL 6-OHDA (Sigma, St. Louis, MO, USA) into the right striatum[39]. After 2 weeks, the surviving PD model rats were peritoneally injected with apomorphine (0.5 mg/kg; Sigma) to test their behavior, and successful PD model rats were confirmed[28]. Rats in the control group were injected with 10 μL sodium chloride (0.9%) into the right striatum.

Cell transplantation for PD

Two weeks after 6-OHDA injection, successfully modeled rats in the BMSC and TH-NTN-BMSC groups were transplanted with 5 μL of BMSC or TH-NTN-BMSC cell suspension (1×10^3 cells/μL) into the right striatum. BMSCs and TH-NTN-BMSCs were labeled with 5-bromodeoxyuridine (10 μM; Sigma) 3 days before transplantation[40].

Behavioral assessment after cell transplantation

After BMSC or TH-NTN-BMSC transplantation, model rats in the PD, BMSC and TH-NTN-BMSC groups were peritoneally injected with apomorphine (0.5 mg/kg, Sigma) four times, and subsequently monitored for behavior at 2, 4, 6 and 8 weeks after transplantation. The number of rotations to the uninjured side (left) was recorded for 30 minutes after apomorphine injection.

Immunohistochemistry for TH and NTN expression in the corpus striatum

At 8 weeks after cell transplantation, rats in all groups were deeply anesthetized with chloral hydrate and cardially perfused with 4% paraformaldehyde. Formalin-fixed paraffin-embedded right corpus striatum tissue (1 mm^3) was cut into 4-μm-thick sections, deparaffinized in three changes of xylene, and rehydrated through a graded alcohol:water series. Antigen retrieval was performed in 10 mM of sodium citrate buffer at pH 6 in an automated pressure cooker. The slides were then rinsed in running water for 5 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide in water for 15 minutes at room temperature. The slides were then rinsed in water and immersed in PBS for 15 minutes at room temperature, followed by blocking in PBS with 5% goat serum (Vector Laboratories, Hamilton, Canada) for 1 hour. Sections were then incubated with mouse anti-rat TH monoclonal antibody (1:500; sc-47708, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-rat NTN monoclonal antibody (1:400; ab49203, Abcam, London, UK) diluted in blocking solution and incubated overnight in a humidified chamber at 4°C. After washing three times with PBS, the slides were incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG at a

dilution of 1:500 for 30 minutes at room temperature (Zymed, San Diego, CA, USA). Diaminobenzidine was used as the chromogen substrate, and hematoxylin was used as the nuclear counterstain. The primary antibody was replaced with PBS or normal rabbit serum (1:100) as a negative control. Slides were imaged by light microscopy (Philips, Amsterdam, Netherlands) and labeled TH and NTN intensity scores were quantified by software Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Western blot analysis of TH and NTN protein expression in the right striatum

Pre-cleared right striatum brain tissue lysates were boiled at 100°C for 5 minutes in SDS-loading buffer. Equal amounts of protein per sample were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in PBST for 2 hours at room temperature and reacted with the primary antibodies (diluted to 0.5 μg/mL in PBST/5% milk; same antibodies as for immunohistochemistry) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies (same antibodies as for immunohistochemistry; 1:2 000) for 45 minutes at 37°C Immunoreactive bands were visualized using enhanced chemiluminescence (GE Healthcare, London, UK). Bands were analyzed using Typhoon 9410 and ImageQuant TL v2008.01 (GE Healthcare).

High performance liquid electrochemical detection of DA and DOPAC content in striatum

Brain samples from the left or right hemispheres containing striatum and substantia nigra were hydrolyzed in a high performance liquid electrochemical measurement system[41]. DA and DOPAC in the left and right corpus striatum were detected and recorded by the high performance liquid electrochemical measurement system.

Ultrastructure of the striatum of model rats observed by post-embedding immunogold electron microscopy

The procedure for post-embedding immunogold electron microscopy was adapted from Bergersen and colleagues[42]. Small rectangular pieces from the corpus striatum region were typically 0.5 mm × 0.5 mm × 1 mm. In the control group, the samples were picked from the left corpus striatum (non-operated and without 6-OHDA injection) in PD model rats. In the PD, BMSC and TH-NTN-BMSC groups, the samples were taken from the right striatum (6-OHDA injection and cell transplantation side). All samples were obtained at 8 weeks after cell transplantation. Ultrathin sections (5 μm) were incubated overnight with primary antibodies against the dopamine D1 receptor (Abcam-ab20066, London, UK) or dopamine D2 receptor (Abcam- ab21218, UK) diluted in TBST containing 2% HAS. The concentrations of the dopamine receptor antibodies were 5 μg/mL. Bound antibodies were visualized by incubating for 2 hours with goat anti-rabbit immunoglobulin secondary conjugated with 10-nm (diameter) colloidal gold (1:20; British Biocell International, London, UK). Sections were observed in a Philips CM20 electron microscope. Images were taken randomly at a primary magnification of × 43 000. Ten individual synapses with clearly defined postsynaptic densities were imaged from each animal. The lengths (nm) of the postsynaptic membranes were marked, and the densities of gold particles were calculated as previously described[42]. For density calculations, gold particles situated within 25 nm (*i.e.*, approximately the same distance as the lateral resolution of the immunogold method)[42] on either side of the midline of the membranes were included in the analysis, and the area containing gold particles was assumed to be a rectangle with a 50-nm width.

Statistical analysis

All quantitative data were presented as mean ± SD, and statistical significance was determined using one-way analysis of variance, unless otherwise stated. The null hypothesis was rejected at the 0.05 level (IBM SPSS Statistics 19.0, IBM, San Francisco, CA, USA).

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Author contributions: Yue Huang participated in animal surgery and data processing. Cheng Chang was responsible for study design and manuscript writing. Jiewen Zhang had full access to all data and participated in data integrity and data accuracy analysis. Xiaoqun Gao was in charge of data collection and study supervision. All authors approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Ethics Committee of Zhengzhou University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent

application/funding source disputations.

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