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Bone Marrow Stromal Cells Express Neural Phenotypes *in vitro* and Migrate in Brain After Transplantation *in vivo*[1]

LI-YE YANG, TIAN-HUA HUANG*, AND LIAN MA

Department of Biology, Medical College of Shantou University, Shantou 515031, Guangdong, China

Objective To investigate the differentiation of bone marrow stromal cells (BMSC) into neuron-like cells and to explore their potential use for neural transplantation. **Methods** BMSC from rats and adult humans were cultured in serum-containing media. *Salvia miltiorrhiza* was used to induce human BMSC (hBMSC) to differentiate. BMSC were identified with immunocytochemistry. Semi-quantitative RT-PCR was used to examine mRNA expression of neurofilament1 (NF1), nestin and neuron-specific enolase (NSE) in rat BMSC (rBMSC). Rat BMSC labelled by Hoschst33258 were transplanted into striatum of rats to trace migration and distribution. **Results** rBMSC expressed NSE, NF1 and nestin mRNA, and NF1 mRNA and expression was increased with induction of *Salvia miltiorrhiza*. A small number of hBMSC were stained by anti-nestin, anti-GFAP and anti-S100. *Salvia miltiorrhiza* could induce hBMSC to differentiate into neuron-like cells. Some differentiated neuron-like cells, that expressed NSE, beta-tubulin and NF-200, showed typical neuron morphology, but some neuron-like cells also expressed alpha smooth muscle protein, making their neuron identification complicated. rBMSC could migrate and adapted in the host brains after being transplanted. **Conclusion** Bone marrow stromal cells could express phenotypes of neurons, and *Salvia miltiorrhiza* could induce hBMSC to differentiate into neuron-like cells. If BMSC could be converted into neurons instead of mesenchymal derivatives, they would be an abundant and accessible cellular source to treat a variety of neurological diseases.

Key words: Bone marrow stromal cell; Cell transplantation; Differentiation; Neuron; Stem cell; *Salvia miltiorrhiza*

INTRODUCTION

Over the past few decades, cell transplantation for treating central nervous system disorders has achieved great success in clinical investigation[1]. When patients with Parkinson's disease were treated with mesencephalic cells from 6- to 10-week-old abortuses, some of them had significant improvement in both clinical symptoms and synthesis of dopamine[1]. However, obtaining the fetal tissue has presented major legal and ethical barriers. In addition, only about 5%-10% of dopaminergic neurons survived, apparently because of immune reactions[1]. For these reasons, attempts have been made to seek alternative cells such as those from bone marrow. A subclass of bone marrow stem cells is capable of differentiating into osteogenic, chondrogenic, adipogenic, and other mesenchymal lineages *in vitro*. They are termed as bone marrow stromal cells (BMSC), or colony-forming-unit fibroblasts, or mesenchymal stem cells. Although BMSC can naturally be a source of surrounding tissues of bone, cartilage, and fat[2-3], several recent reports have demonstrated that these cells, under specific experimental conditions, can differentiate into muscle, glia, and hepatocytes[4-9]. The present study was therefore carried out further to test if BMSC could differentiate into neurons and glia both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)/ F-12 medium, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin and L-glutamine were obtained from GIBCO/BRL (Boston, USA). Recombinant human basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and nerve growth factor (NGF) were purchased from Sigma (USA). The following antibodies were obtained and

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*Correspondence should be addressed to Tian-Hua HUANG, Department of Biology, Medical College of Shantou University, Shantou 515031, Guangdong, China. Tel: 86-754-8900442. Fax: 86-754-8557562. E-mail:yangleeyee@sina.com

Biographical note of the first author: Li-Ye YANG, male, born in 1969, associate professor, majoring in stem cells and brain repair.

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diluted: mouse monoclonal anti-vimentin (1:150, Zymed, USA), rabbit polyclonal anti-nestin (1:100, Boster, Wuhan, China), mouse monoclonal anti-NSE (neuron specific enolase, 1:100, Zymed, USA), mouse monoclonal anti-NF200 (neurofilament 200, 1：100，Zymed, USA), mouse monoclonal anti-GFAP (glial fibrillary acidic protein, 1:100, Zymed, USA), mouse monoclonal anti-S100 (1:100, Zymed, USA), mouse monoclonal anti-alpha smooth muscle protein (alpha-SMA, 1:100, Zymed, USA), mouse monoclonal anti-CD34 and CD31 (1:100, Zymed, USA). SP-9000 immunocytochemistry kits were from Beijing Zhongshan Corp (Beijing, China), which contained biotin labelled goat anti-mouse IgG and goat anti-rabbit IgG, HRP labelled streptavidin and avidin biotin blocking system. AEC was from Beijing Zhongshan Corp, culture dishes and plates were from Nunc (Denmark). *Salvia miltiorrhiza* was ontained from Shanghai Sino-Western Pharmarco. Corp (Shanghai, China). *Bis*-benzamide was purchased from Sigma.

Primary Bone Marrow Stromal Cell Cultures

For human BMSC (hBMSC) culture, bone marrow from healthy donors (n=3) was aspirated, and Hanks solution was added into bone marrow and centrifuged at 500 g for 10 min. The supernatant was discarded, and mononuclear cells were transferred to culture plates or dishes containing DMEM/F12 (1:1), 10% FBS, 20 ng/mL bFGF, 20 ng/mL EGF, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin. The cells were plated at a density of 10 000 cells/cm^2. After 48 h, media were changed, at which point adherent cells were scarce. The cells were re-fed every 2-3 days as necessary. After the cells grew to near confluency, they were detached by 0.25% trypsin/1 mmol/L EDTA for 3-4 min at 37℃, and diluted 2-3 times，passaged into flasks or dishes and same culture medium was added.

Rat BMSC (rBMSC) were cultured as previously described[10]. In brief, adult male Sprague-Dawley rats (Shanghai Experimental Animal Center, Shanghai, China) were anaesthetized with pentabarbital, epiphyses of femurs and tibiae were removed, and the bone marrow was flushed out using a syringe filled with medium. The cells were disaggregated by gentle pipetting several times. The bone marrow was filtered through a 70-μm nylon mesh to remove remaining clumps of tissue. The cells were washed by adding buffer, centrifuged for 10 min at 200 g and the supernatant was removed and plated in 75-cm^2 flasks with DMEM containing 20% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 ng/mL amphotericin B. About 24 h after plating, the supernatant containing nonadherent cells was removed and fresh medium was added. After the cells grew to near confluency, they were passaged 2-5 times by being detached (0.25% trypsin/1 mmol/L EDTA for 5 min) and replated at a density of 5 000 cells/cm^2.

Salvia Miltiorrhiza Induced hBMSC to Differentiate into Neuron-like Cells

Passaged cells of hBMSC (passage 1 to passage 3) were plated on dishes and maintained in subconfluent state. To induce neuronal differentiation, the media were removed, and the cells were washed with PBS and transferred to the neuronal induction media composed of DMEM/F12/N2/2% (vol/vol) *Salvia miltiorrhiza*. Under these conditions, the cells could differentiate into neuron-like cells. The cells were fixed for immunocytochemical examination at different time points ranging from 3-10 days post-induction.

To induce neuronal differentiation of rBMSC, passaged cells of rBMSC (passage 3) were plated in 75-cm^2 flasks and maintained in subconfluent state. Twenty-four hours prior to the neuronal induction, media were replaced by pre-induction media consisting of DMEM/20%FBS/20 ng/mL bFGF. To induce neuronal differentiation, the pre-induction media were removed, and the cells were washed with Hanks solution and transferred to neuronal induction media composed of DMEM/2% (vol/vol) *Salvia miltiorrhiza*. Under these conditions, the cells differentiated into neuron-like cells. Twenty-four hours later, mRNA was extracted and PCR was performed with the non-induced rBMSC being served as the control.

RT-PCR

Total RNA was extracted from rBMSC that were undifferentiated or had been differentiated for 24 hours after *Salvia miltiorrhiza* induction, using TRIzol RNA isolation reagents (Life Technologies, Shenzhen, China). RNA concentration was determined spectrophotometrically. Primers of NSE, NF1, GAPDH and nestin were the same as previously described[11]. Reverse transcription reaction and semi-quantitative RT-PCR were performed under the same conditions[11]. PCR product was subject to electrophoresis on 1.5% agarose gels and then photographed.

Maintenance of Long-term Neuronal Cultures

To promote long-term survival of hBMSC-derived neurons, several additional components were added to the neuronal induction media. Long-term

neuronal induction media consisted of DMEM/F12/B27, 20 ng/mL bFGF, 20 ng/mL EGF and 100 ng/mL NGF. Under these conditions, the differentiated neuron-like cells could survive for a long term.

Immunocytochemical Examination and Quantification of Neuronal Differentiation

For the immunoperoxidase method, a commercial kit (SP-9000) was used. The cells were fixed on culture dishes by treating with 4% buffered paraformaldehyde for 10 min. The samples were blocked with 5% normal blocking serum (derived from same species as the secondary antibody) in PBS for 30 minutes, and incubated with primary antibody for 60 minutes at room temperature. The cells were incubated with secondary antibody for 15 min and washed 3 times with PBS, then incubated with peroxidase-linked complex for 15 min. After washed with PBS, the cells were developed by AEC substrate according to kit instructions. Sometimes samples were counterstained with hematoxylin. Three independent experiments were performed in duplicate. For quantification, a camera was used to capture 10 non-overlapping low power image (×200) of each marker. The cells with positive staining were counted as percentage of total cell counts. The data were expressed as $\bar{x} \pm s$ deviation.

Intracerebral Transplantation and Brain Tissue Processing

To label the nuclei of rBMSC (passage 2) fluorescently, the cells were incubated with 1 μg/mL *bis*-benzamide for 24 hours before implantation. Adult Sprague-Dawley rats (200-300 g) were anesthetized by intraperitoneal injection of 350 mg/kg of chloral hydrate and the animals were transferred to a stereotactic apparatus in clean field. A 2- to 5-mm incision was made in the scalp 2 mm lateral to the bregma. A burr hole was made in the bone 3 mm lateral to the bregma, and about 4 μL of the cell suspension was slowly injected for 5 min into the striatum at a depth of 4-5 mm from the surface of the brain. The wound was closed with interrupted surgical sutures. After 1, 2, and 3 months (3 rats, respectively), rats were sacrificed by intracardiac perfusion under deep anesthesia with ice-cold PBS, followed by 3% bufferd paraformaldehyde then by 10% sucrose. The brains were removed, the forebrain was trimmed, 20 micron tissue sections were prepared with a cryostat. Frozen sections were attached to gelatin-coated slides and quickly immersed in cold acetone and stored at -20℃ for further processing. The transplant site was located by microscopically identifying the fluorescently labelled cells in the tissue section. Fluorescently labelled cells was visualized and photographed using a fluorescent microscope. The number of fluorescently labelled cells were visualized and photographed. The number of fluorescently labelled nuclei was counted in 8-10 sections cut from rostal to caudal limit of the stratum. The procedure was repeated on each brain by two individuals. Only the clearly labelled nuclei were counted. Dead and lysed cells left a bluish hue in the surrounding tissue and no clear staining.

RESULTS

BMSCs Culture and Proliferation

Human BMSC were successfully isolated from adult human bone marrow and expanded as primary cultures. Only a small number of cells were adherent in primary culture. At low plating densities, hBMSC grew as a monolayer of large, flat cells with a vesicular nucleus and multiple long processes reminiscent of fibroblast cells. As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology (Fig. 1). hBMSC could double in 2-3 days stimulated by bFGF and EGF, and could be steadily cultured and passaged *in vitro* for a long term. In one case, hBMSC were passaged 10 times for 40-day culture *in vitro*, and proliferated about 6×10^7 times, but their proliferation rate did not apparently slow down. rBMSC were cultured and their proliferation and characteristics were reported previously[10].

hBMSCs Expressed Neural Phenotypes

Immunocytochemical staining showed that the majority of the isolated cells were mesenchymal origin, and expressed vimentin, which was the marker for mesodermal cells (Fig. 2). hBMSCs were not stained by CD34 and CD31, indicating that there were no hemopoietic stem cells and endothelial cells in BMSC. About 0.01%-0.02% of the total cells were stained heavily by nestin, and the positive cells were flat, irregular or multiangular (Fig. 3). Most of the cells were stained by NSE, and the positive sites were in cytoplasm (Fig. 4). About 40%-50% cells expressed NF-200, and the positive cells were fibroblast-like, some cells with big and flat body were stained heavily (Fig. 5). About 60% hBMSC were stained by beta-tubulin (Fig. 6), while 0.1%-0.2% of the total cells were stained heavily by S100, and the positive sites were in cytoplasm, these cells were flat and irregular (Fig. 7). Strong GFAP-positive cells could be detected (Fig. 8), but they were quantitatively minor (<0.001%). Interestingly, 20% of hBMSC expressed alpha smooth muscle protein, indicating the existence of smooth muscle cells (Fig. 9).

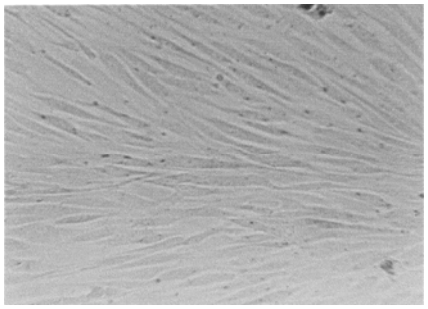

FIG. 1. Cultured primary hBMSC, phase contrast microscopy 80×.

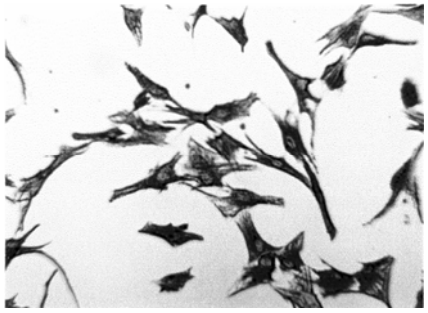

FIG. 2. Vimentin expression in hBMSC, original magnification 80×.

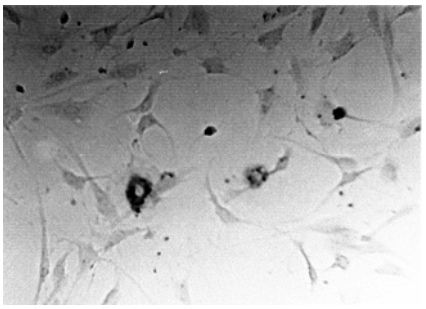

FIG. 3. Nestin-stained cells in hBMSC (passage 1). original magnification 80×.

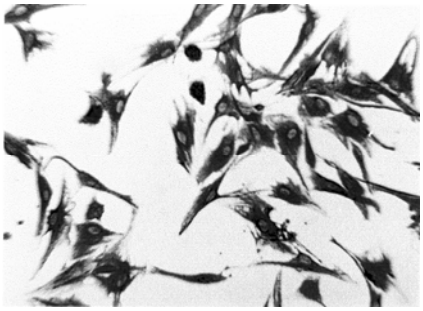

FIG. 4. NSE expression in hBMSC (passage 1). original magnification 80×.

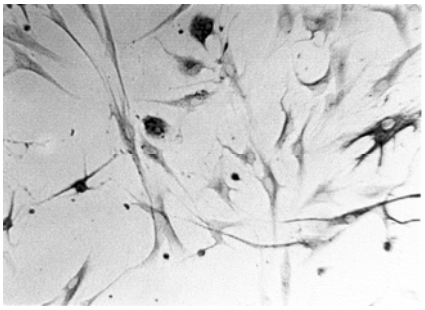

FIG. 5. NF200 expression in hBMSC (passage 1). original magnification 80×.

FIG. 6. Beta-tubulin Ⅲ expression in hBMSC (passage 1). original magnification 80×.

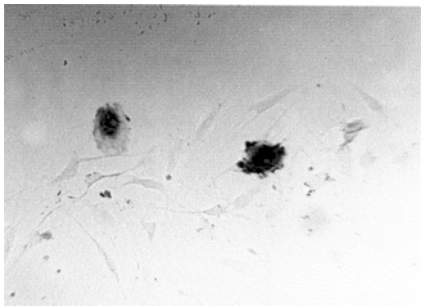

FIG. 7. S100 positive cells in hBMSC (passage 1). original magnification 80×.

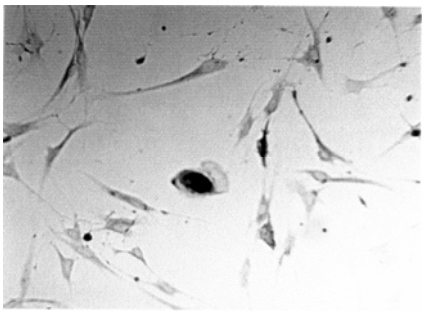

FIG. 8. A GFAP-positive cells in hBMSC, original magnification 80×.

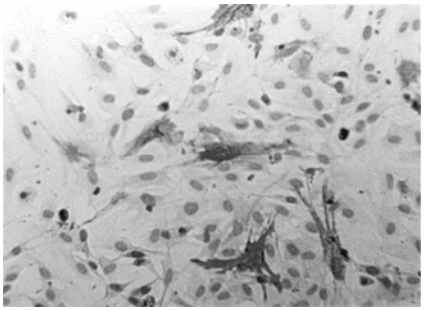

FIG. 9. Differentiation of hBMSC into smooth muscle cells and expression of alpha-SMA, original magnification 80×.

Morphological Change of hBMSC by Salvia miltiorrhiza Induction

To induce neuronal differentiation, the cells were transferred and incubated in a serum-free medium containing 2% (vol/vol) *Salvia miltiorrhiza* for 24 h. Change in morphology of some BMSC was apparent. The responsive cells progressively assumed neuronal morphological characteristics. Initially, the cytoplasm in the flat BMSC retracted to the nuclei, forming a contracted multipolar cell body. During the subsequent 24 h, cell bodies became increasingly spherical and refractile, displaying primary and secondary branches (Fig. 10). To promote long-term survival of hBMSCs-derived neurons, the neuron-like cells were transferred into a new medium after 48h induction. Long-term neuronal induction media consisted of DMEM/F12/B27, 20 ng/mL bFGF, 20 ng/mL EGF and 100 ng/mL NGF. Under these conditions, differentiated neuron-like cells could survive for a long term.

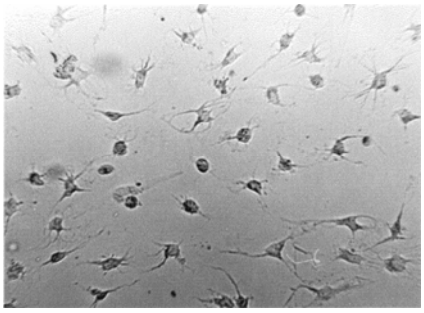

FIG. 10. Differentiation of hBMSC into neuron-like cells induced by *Salvia miltiorrhiza,* original magnification 80×.

Immunocytochemical Identification of Differentiated Cells

To further characterize neuronal differentiation, we fixed *Salvia miltiorrhiza*-treated cultures after 3 days and stained them for neuronal markers NSE, NF200 and beta-tubulin Ⅲ. The expression pattern of three markers (percentage) was very similar to that of undifferentiated hBMSCs. Some (not all) new generated neuron-like cells expressed three markers for neuron, but some unresponsive flat hBMSC also expressed beta-tubulin Ⅲ, NF200 and NSE protein. The contracted cell bodies elaborated processes and were stained intensely for beta-tubulin Ⅲ (Fig. 11). After *Salvia miltiorrhiza* treatment, we could also identify nestin, GFAP and S100 positive cells. Interestingly, some new generated neuron-like cells expressed SMA (Fig. 12).

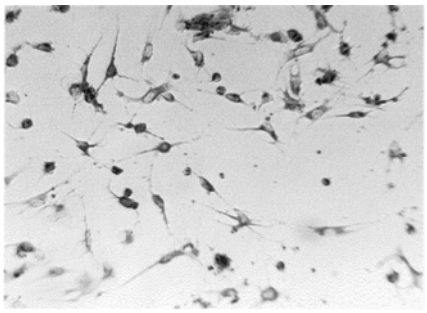

FIG. 11. hBMSC derived neurons heavily stained by beta-tubulin Ⅲ original magnification 80×.

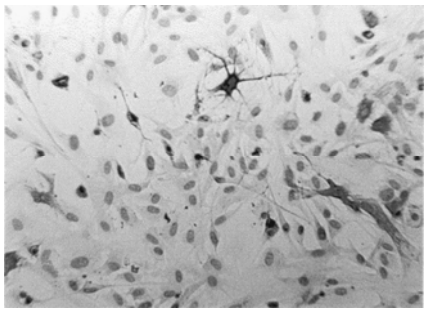

FIG. 12. Spontaneous differention of hBMSC into neuron-like cells, and a neuron-like cells heavily stained by alpha-SMA. original magnification 80×.

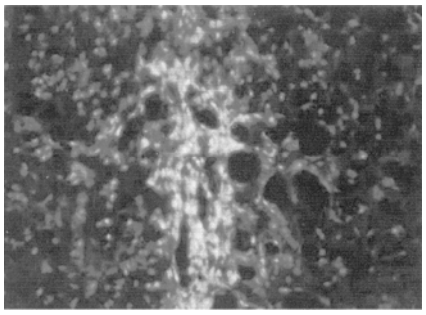

FIG. 13. Tranplantation of Hoschst 33258-labelled rBMSC into striatum of adult rats, and extensively migrated cells fluorescence microscope 160×.

RT-PCR Results of rBMSCs

RT-PCR amplification indicated that rBMSC expressed NSE and nestin mRNA, but only weak expression of NF1 mRNA was detected. After *Salvia miltiorrhiza* induction for 24 hours, NSE and nestin expression maintained the same level, but NF1 expression increased apparently compared to the control (Fig. 14). rBMSCs could also differentiate into neuron-like cells and express neuron phenotype as previously described.

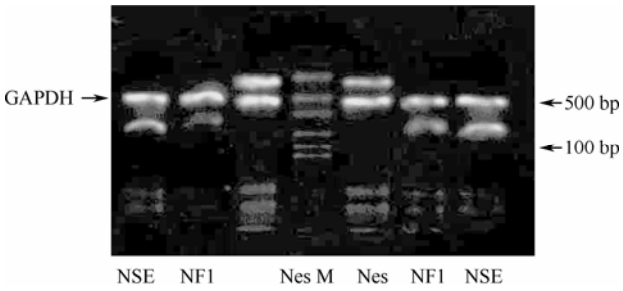

FIG. 14. RT-PCR results of rBMSC. Lane M: Marker, 3 left lanes: RT-PCR results of rBMSC, 3 right lanes: mRNA expression 24 hours after *Salvia miltiorrhiza* induction.

Survival and Migration of the Implanted Cells After Injection in the Striatum

rBMSC were injected into the corpus striatum of the brains. After 1, 2, 3 months, the rats were killed and the brains were dissected and stained with hemotoxylin and eosin, finding that there was no significant gliosis or infiltration of leukocytes around the implantation site (data not shown). Fluorescently labeled cells were readily detected in the brain sections. About 40000-62000 of rBMSC were present in the brains. Because the number of cells injected varied from 100 000 to 120 000, about 40% of the transplanted rBMSC were recovered after 1 month.

The transplanted cells were found in multiple areas of the brain including the cortex. The cells persisted in the sites to where they migrated for 1 month. The highest concentration of cells was found around the rotrocaudal axis in the striatum and along the corpus callosum (Fig. 13). There were fewer cells in the cerebral cortex. Clusters of labeled cells were observed in the temporal lobe regions at all time points examined. At month 3, fewer cells were found in the outlying cortical regions, suggesting that the cell number was decreased between months 2 and 3.

DISCUSSION

The present study demonstrated that hBMSC could express beta-tubulin Ⅲ, NSE and neurofilament 200 of neuronal phenotyte *in vitro*, and *Salvia miltiorrhiza* could induce BMSC to differentiate into neuron-like cells. Morphological characteristics of rBMSC transplanted into adult rat brains were similar to those of astrocytes, suggesting that the transplanted cells could migrate a long distance and adapt well in the host brain. Together with other researches, our data indicated that stem cells from bone marrow are capable of differentiating into neurons[7-10,12-13], but it is not clear whether these differentiated neurons are functional or had neurotransmitter-secreting ability.

Salvia miltiorrhiza could induce neuronal differentiation of BMSC. However, the mechanism responsible for its inducing activity remains unclear. It has been shown that, three water-soluble components identified previously in *Salvia miltiorrhiza* had antioxidant activity[14]. Fe(2+)-cysteine could induce lipid peroxidation (malondialdehyde formation), and decrease the production of superoxide anion radical (O^{2-}) in xanthine-xanthine oxidase system. These components may protect neuron-like cells from differentiating into BMSC through the mechanism similar to that of β-mercaptoethanol and DMSO[7].

The immunocytochemical examination identified that 0.1%-0.2% of BMSC were positive for S100, but type Ⅱ collagen positive cells were not found in hBMSC, indicating that S100 positive cells were not chondrocytes, because chondrocytes could be stained by S100. Most likely, S100-positive cells were Schwann cells. It was reported that rBMSC and bone marrow cells could myelinate injurious peripheral nerves *in vivo*, new generated myelins were functional and myelin cells were immunoreactive with S100, the marker for Schwann cells[15-16]. In our research, Schwann-like cells from hBMSC only expressed S100, but not GFAP. This was different from that of Schwann cells from peripheral nerves which were immunoreactive with S100 and GFAP. Different tissue sources might account for this discrepancy. Further study is needed concerning whether these kinds of S100-positive cells could form myelin.

Previous *in vivo* studies in mice demonstrated

that cells from bone marrow express neuronal phenotypes[12-13], while researches in humans showed that transplanted bone marrow could generate new neurons in human brains[17-18]. But neurons derived from bone marrow lacked the typical morphology of neurons, and cell fusion might be responsible for some trans-differentiation[19]. It was shown that BMSC transplanted into brain and injurious spinal cord, promoted the functional recovery of diseased CNS[20-21], but they did not secret neurotransmitter and had the electrical properties of neurons. The present study showed that some neuron-like cells expressed alpha smooth muscle actin, but full characteristics of neurons from central nervous system were not seen. It was therefore concluded that some neuron-like cells observed in the present study expressed only some proteins of neurons but they had not become functional neurons yet.

Cell replacement by transplantation of human fetal dopamine neurons has been proved to be effective in long-term treatment of central nervous system disorders in clinical trials[1]. However, substantial ethical and practical concerns about the use of human fetal tissues have precluded their widespread application. Cells from bone marrow could differentiate into neurons. This could be an alternative to cell therapy of the nervous system by eliminating the need for harvesting autologous human neural stem cells. In fact, BMSC could grow rapidly in culture. Therefore, massive BMSC can be generated from 1 mL of bone marrow aspirate if the incubation conditions are well optimized. If marrow stromal stem cells represent just a special case of post-natal multipotent stem cells, there is little doubt that they represent one of the most accessible sources of such cells for therapeutic use. The ease with which they are harvested (a simple marrow aspirate), and the simplicity of the procedure required for their culture and expansion *in vitro* may make them an ideal candidate[22].

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