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Neuronal-like cell differentiation of non-adherent bone marrow cell-derived mesenchymal stem cells*

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Research Highlights

- (1) Mouse non-adherent bone marrow-derived mesenchymal stem cells developed colony-forming unit-fibroblasts *in vitro*, and could differentiate into neuronal-like cells *in vivo* and *in vitro*.
- (2) Epidermal growth factor promoted the growth of mouse non-adherent bone marrow-derived mesenchymal stem cells.
- (3) Non-adherent bone marrow-derived mesenchymal stem cells can be used as seed cells for the treatment of nervous system diseases.

Abstract

Non-adherent bone marrow cell-derived mesenchymal stem cells from C57BL/6J mice were separated and cultured using the "pour-off" method. Non-adherent bone marrow cell-derived mesenchymal stem cells developed colony-forming unit-fibroblasts, and could be expanded by supplementation with epidermal growth factor. Immunocytochemistry showed that the non-adherent bone marrow cell-derived mesenchymal stem cells exposed to basic fibroblast growth factor/epidermal growth factor/nerve growth factor expressed the neuron specific markers, neurofilament-200 and NeuN, *in vitro*. Non-adherent bone marrow cell-derived mesenchymal stem cells from β -galactosidase transgenic mice were also transplanted into focal ischemic brain (right corpus striatum) of C57BL/6J mice. At 8 weeks, cells positive for LacZ and β -galactosidase staining were observed in the ischemic tissues, and cells co-labeled with both β -galactosidase and NeuN were seen by double immunohistochemical staining. These findings suggest that the non-adherent bone marrow cell-derived mesenchymal stem cells could differentiate into neuronal-like cells *in vitro* and *in vivo*.

Key Words

neural regeneration; stem cells; non-adherent bone marrow cell-derived mesenchymal stem cells; neuronal-like cells; colony-forming unit-fibroblasts; proliferation; differentiation; beta-galactosidase transgenic mouse; cell transplantation; cerebral ischemia; bone marrow cells-derived mesenchymal stem cells; grants-supported paper; neuroregeneration

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INTRODUCTION

Stem cell therapy holds great promise for the treatment of degenerative or inherited diseases because stem cells undergo self-renewal and differentiation into multiple cell types^[1]. Compared with embryonic stem cells, bone marrow-derived mesenchymal stem cells (MSCs) are one of the most promising types of stem cell for transplantation therapy because they are easily isolated, simple to expand *in vitro*, amenable to genetic manipulation and maintain their pluripotency after repeated passages^[2]. However, despite their extensive *in vitro* characterization and *in vivo* therapeutic potential, relatively little has been reported with respect to their inherent biology *in vivo*.

MSCs were previously referred to as “marrow stromal cells” or “colony-forming unit-fibroblasts” (CFU-fs), which reflect their origin and morphology in culture. Based on their pioneering studies initiated nearly 40 years ago, Friedenstein *et al*^[3] were the first to propose the concept that human postnatal bone marrow contained a precursor cell for multiple mesenchymal cell lineages. Over the ensuing decades, marrow stromal cells have been characterized based largely upon their properties *in vitro* or following transplantation in various animal model systems^[4-6]. The term CFU-f was coined by Friedenstein to describe cells isolated from the bone marrow stroma of a variety of postnatal organisms that are adherent, fibroblastic and clonogenic in nature^[7]. Under well-defined *in vitro* and *in vivo* conditions, a proportion of CFU-fs can give rise to multiple mesenchymal tissues, including bone, adipose tissue, cartilage, myelosupportive stroma, smooth muscle, cardiomyocytes and tendon. Recent studies have shown that adult bone marrow-derived MSCs differentiate not only into mesenchymal cells, but also into cells with the characteristics of visceral mesoderm, neuroectoderm and endoderm *in vitro* and *in vivo*^[8-9].

MSCs (CFU-fs) were defined as highly adherent fibroblastic cells^[10]. Rickard and colleagues^[11] also emphasized that all of the

stromal cells in primary rat bone marrow cells cultures had attached within 24 hours. Such findings have led many scientists to believe that CFU-fs are highly adherent fibroblastic cells, which resulted in limited studies on the possible non-adherent nature of MSCs. Nevertheless, a few reports have shown that the non-adherent bone marrow cells can give rise to CFU-fs *in vitro*^[12-15] and form skeletal muscles^[16] and bone^[17] after *in vivo* transplantation. Recent evidence has suggested the existence of a type of non-adherent MSC in adult bone marrow^[18]. When the non-adherent bone marrow cells are transferred daily into fresh dishes, they can develop CFU-fs continuously^[15, 19]. After exposure to induction medium, they also can differentiate into adipocytes, osteoblasts, chondrocytes and glia cells^[20]. However, it is unknown whether they can differentiate into neuronal cells *in vitro* or *in vivo* and whether they can be used as candidates for transplantation therapy for neurological diseases.

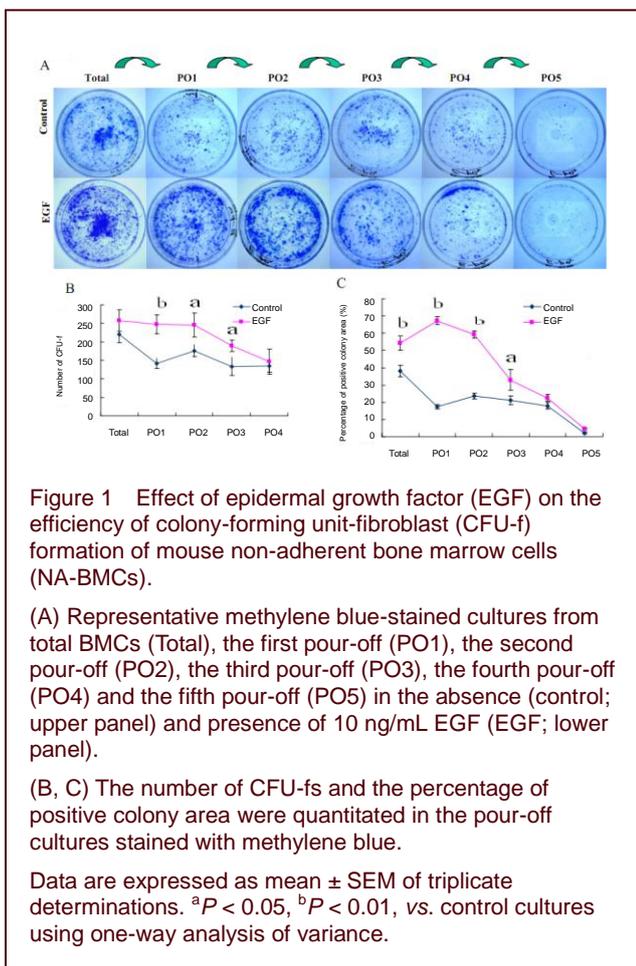
In the present study, we investigated whether mouse non-adherent bone marrow cells could give rise to CFU-fs, whether epithelial growth factor could stimulate the formation of CFU-fs by the non-adherent bone marrow cells, and whether mouse non-adherent bone marrow cell-derived MSCs could differentiate into neuronal cells *in vitro* and *in vivo*.

RESULTS

Epidermal growth factor promoted CFU-f formation by mouse non-adherent bone marrow cells

Total mouse bone marrow cells (the cells obtained on the first day after removing the non-adherent bone marrow MSCs) gave rise to CFU-fs, and the “pour off” non-adherent bone marrow cells also gave rise to CFU-fs. This was continuously demonstrable for at least the first five transfers, and there was usually a sequential decrease in the number of CFU-fs in the supernatants of the pour-offs (Figure 1A, upper panel, and B). Treatment with epidermal growth factor also

led to a significant increase in the number of CFU-fs and the positive colony area of the total bone marrow cells in the pour-off 1–4 cultures (Figure 1; $P < 0.05$ or 0.01). The frequency of CFU-f formation was increased by 1.2, 1.8, 1.4, and 1.4 fold in the total bone marrow cells and the pour-off 1, 2 and 3 cultures, respectively, in the epidermal growth factor treated cultures compared with the control cultures (Figure 1B). The positive colony area was increased 1.54, 4.25, 2.51 and 1.56 fold in the total bone marrow cells and the pour-off 1, 2 and 3 cultures, respectively, in the epidermal growth factor treated cultures compared with the control cultures (Figure 1C). Consequently, treatment with epidermal growth factor not only increased the number of CFU-fs, but also enhanced the proliferation of adherent bone marrow-derived MSCs.



Non-adherent bone marrow cell-derived MSCs differentiated into neuronal-like cells *in vitro*

Immunocytochemistry showed that after 2 weeks of culture with human epidermal growth factor/human basic fibroblast growth factor/human nerve growth factor, the cells from both total bone marrow cell-derived and the non-adherent bone marrow cell-derived MSCs displayed morphological features of nerve cells, with long mul-

ti-polar extensions and branching ends, and expressed neurofilament-200 in the cytoplasm (Figure 2A and B) and NeuN in the nucleus (Figure 2C and D). Consequently, both total bone marrow cell-derived and the non-adherent bone marrow cell-derived MSCs could be induced to differentiate into neuronal-like cells.

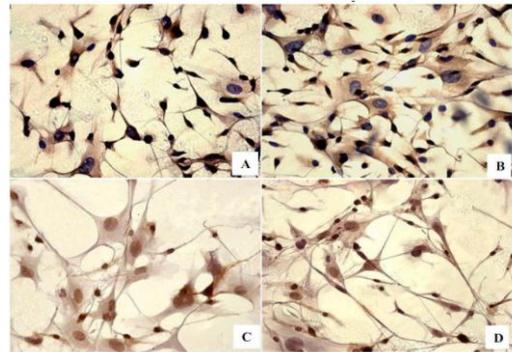


Figure 2 Mouse non-adherent bone marrow cell (NA-BMC)-derived mesenchymal stem cells (MSCs) differentiated into neuronal-like cells similar to total BMC-derived MSCs *in vitro* (immunocytochemical staining, $\times 400$)

The total BMC-derived and the NA-BMC-derived MSCs were cultured with human epidermal growth factor/human basic fibroblast growth factor/human nerve growth factor for 2 weeks and the expression of neural-specific antigen, neurofilament-200 (NF-200) and NeuN was assessed by immunocytochemistry.

Representative micrographs of induced cells stained immunocytochemically for NF-200 from the total BMC-derived MSCs (A) and from the NA-BMC-derived MSCs (B) showing brown positive staining in the cytoplasm of induced cells.

Representative micrographs of induced cells stained immunocytochemically for NeuN from the total BMC-derived MSCs (C) and from the NA-BMC-derived MSCs (D) showing brown positive staining in the nuclei of induced cells.

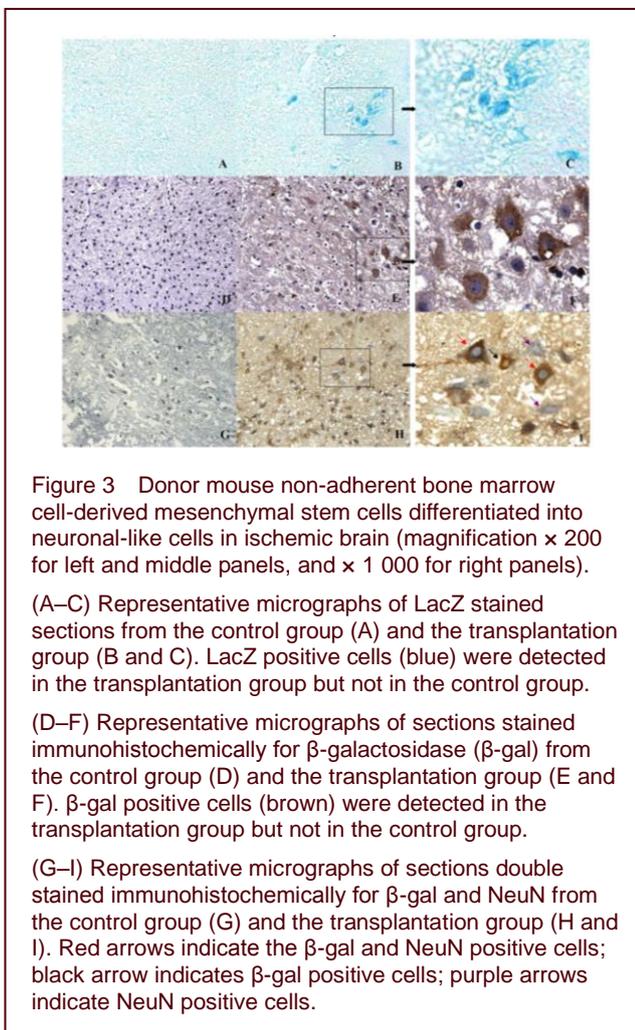
Quantitative analysis of experimental animals

A total of 24 C57BL/6J mice were used for the ischemic brain model. The successful model mice were randomly assigned to transplantation and control groups, which were injected with non-adherent bone marrow MSCs from β -galactosidase transgenic mice or an equivalent volume of PBS into the right corpus striatum, respectively. All 24 mice were included in the final analysis.

Transplanted non-adherent bone marrow cell-derived MSCs differentiated into neuronal-like cells in ischemic brain

Donor cells originating from non-adherent bone marrow cell-derived MSCs were identified by LacZ staining and immunohistochemical staining for β -galactosidase. At 8 weeks, LacZ and β -galactosidase positive cells were

detected only in the transplantation group (Figure 3B, C and E, F, respectively), but not in the control group (Figure 3A, D). In the sections double stained for β -galactosidase and NeuN, three types of cells were observed including β -galactosidase single positive cells (brown cytoplasm, black arrow), NeuN single positive cells (gray nuclei, purple arrow) and cells co-positive for β -galactosidase and NeuN (red arrow) in the transplantation group (Figure 3H, I). However, only single NeuN positive cells were found in the control group (Figure 3G). These results indicate that mouse non-adherent bone marrow cell-derived MSCs could differentiate into neuronal-like cells *in vivo* and contribute to the reconstruction of ischemic brain.



DISCUSSION

To determine whether the mouse non-adherent bone marrow cells could give rise to CFU-fs, and whether epidermal growth factor could stimulate CFU-f formation by mouse non-adherent bone marrow cells, “pour-off”

mouse bone marrow cell cultures were employed and the effect of epidermal growth factor on CFU-f formation was examined. In this study, we demonstrated that the mouse non-adherent bone marrow cells could give rise to CFU-fs, that the frequency of CFU-f formation by the non-adherent bone marrow cells could be enhanced by treatment with epidermal growth factor, and that mouse non-adherent bone marrow cell-derived MSCs could differentiate into neuronal cells *in vitro* and *in vivo*.

The presence of non-adherent MSCs in rat bone marrow cells has been previously demonstrated, and it has been suggested that the transformation from a non-adherent to an adherent phenotype might be involved in the actions of compounds such as prostaglandin E_2 ^[15], $1,25(OH)_2$ vitamin D_3 ^[21] and parathyroid hormone^[22]. Here, we demonstrated that epidermal growth factor can also work on these non-adherent progenitors by stimulating their self-renewal in suspension, their adhesion, and subsequently their proliferation. The expression of epidermal growth factor receptor in bone marrow-derived MSCs has been reported^[23]. Previous studies have demonstrated that epidermal growth factor exerts various actions by binding and activating the epidermal growth factor receptor, including cell migration and proliferation, in a wide variety of cell types^[24-25]. Tamama *et al*^[26] reported that epidermal growth factor stimulated motility in rat and immortalized human bone marrow-derived MSCs. However, epidermal growth factor-induced proliferation was observed in immortalized human bone marrow-derived MSCs but was not apparent in rat bone marrow-derived MSCs^[26]. Our results suggest that epidermal growth factor promoted the self-renewal, adhesion and proliferation of mouse non-adherent bone marrow cell-derived MSCs. Although bone marrow-derived MSCs hold great promise for the regeneration of various tissues following transplantation and as vehicles for gene therapies, a major stumbling block is the low number of such bone marrow-derived MSCs, which necessitates their *ex vivo* expansion. Our findings suggest that epidermal growth factor could be used for the expansion of bone marrow-derived MSCs prior to cell transplantation. Epidermal growth factor may enable the expansion of bone marrow-derived MSCs more effectively *in vitro* to obtain sufficient donor cells for therapeutic transplantation from the limited donor bone marrow.

Recent studies have shown the ability of bone marrow cells to migrate in the brain and to acquire neuronal or glial characteristics. *In vitro*, bone marrow-derived MSCs can be induced with chemical compounds to express markers of these lineages^[27]. However, following chem-

ical treatment with-mercaptoethanol, dimethylsulfoxide and butylated hydroxyanisole, the cells gained neuronal-like morphology within a few hours^[27], which was lost after withdrawal of the inductive conditions. It is very unlikely that changes in cellular organization, gene and protein expression related to differentiation commitment can become so advanced within this short time of exposure to inductive molecules, and these reported changes probably reflect cytotoxicity^[28]. Although it is well recognized that bone marrow-derived MSCs can differentiate into neuronal-like cells when supplemented with growth factors^[29], it remains unknown whether the non-adherent bone marrow cell-derived MSCs can differentiate into neuronal cells. The present study demonstrated that not only the total bone marrow cell-derived MSCs, but also the non-adherent bone marrow cell-derived MSCs, can differentiate into neuronal-like cells when supplemented with epidermal growth factor, basic fibroblast growth factor and nerve growth factor. Under differentiating conditions, both the total bone marrow cell-derived and the non-adherent bone marrow cell-derived MSCs display a distinct neuronal shape and express the neuronal markers, neurofilament-200 and NeuN. Our findings indicate that both total bone marrow cell-derived and the non-adherent bone marrow cell-derived MSCs can be induced to differentiate into neuronal-like cells *in vitro*.

In animal models of neurological disorders for cerebral ischemia, Parkinson's disease and spinal cord lesions, the transplantation of MSCs has been reported to improve functional outcomes. Human MSCs are known to secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities for damaged tissues, including the brain^[30]. Three mechanisms have been suggested for the effects of the MSCs: transdifferentiation of the grafted cells to replace degenerating neural cells, cell fusion, and neuroprotection of the dying cells^[31]. Although the neural differentiation capacity of MSCs *in vitro* has been widely explored, the *in vivo* response of this cell type upon direct engraftment into the brain has not been adequately assessed. Recently, a study demonstrated that non-induced MSCs integrated into the postnatal neurogenic pathway of the RMS/olfactory bulb system by migrating appropriately and differentiating into olfactory granule cells, supporting the conclusion that the bone marrow-derived adult stem cells indeed possess the capacity for neural transdifferentiation under the influence of environmental cues from the brain^[32]. Chen *et al*^[33] transplanted the MSCs into middle cerebral artery occlusion model mice through a vein and found that the donor cells homed to the ischemic brain, suggesting that the homing of MSCs

is regulated by cytokines in the ischemic microenvironment. Using models of middle cerebral artery occlusion and contusion traumatic brain injury, Li and Chopp^[6] demonstrated that MSCs derived from donor rats, mice or humans transplanted into rodent brain intracerebrally, intraarterially, intracisternally, intrathecally or intravenously, significantly improve neurological outcomes. MSCs primed with valproate and/or lithium have been shown to improve functional recovery, reduce brain infarct volume, and enhance angiogenesis in a rat model of middle cerebral artery occlusion^[34]. In this study, we demonstrated that mouse non-adherent bone marrow cell-derived MSCs could differentiate into neuronal-like cells *in vivo*. Non-adherent bone marrow cell-derived MSCs from β -galactosidase transgenic mice were transplanted into focal ischemic wild-type mice and donor cells originating from non-adherent bone marrow cell-derived MSCs were identified by LacZ staining and β -galactosidase immunohistochemistry. The differentiation of the donor cells into neuronal cells was determined by double staining for β -galactosidase and NeuN. LacZ and β -galactosidase positive cells were detected only in the transplanted ischemic side of the brains, but not in the control side of the brains. Cells double positive for β -galactosidase and NeuN were observed in the transplanted ischemic side of the brains, but only single NeuN positive cells were found in the control side of the brains. These results indicate that mouse non-adherent bone marrow cell-derived MSCs could differentiate into neuronal cells *in vivo* and contribute to the ischemic brain reconstruction. This study suggests that the transplantation of non-adherent bone marrow cell-derived MSCs may be used as a cell therapy to treat neurological disorders such as cerebral ischemia.

MATERIALS AND METHODS

Design

Comparative observation of cytology and randomized, controlled, animal study.

Time and setting

The experiments were conducted in the Nanjing Medical University, China, from June to December 2011.

Materials

A total of 50 male C57BL/6J mice, aged 4 weeks, weighing 22 ± 2 g, were used. Twenty-six mice were sacrificed for collecting MSCs, and the remaining 24 mice were prepared as models of brain ischemia for *in vivo* experiments. All animals were purchased from the

Animal Center of Nanjing Medical University (SCXK (Su) 2005-002). Another five male β -galactosidase transgenic mice (129-Gt (ROSA) 26Sor/J, 4 weeks old, 22 ± 2 g) were purchased from Jackson Laboratory, Toronto, Canada, for cell transplantation. All animal experiments were conducted in compliance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[35].

Methods

Culture of non-adherent bone marrow cells and formation of CFU-fs

Tibiae and femurs were removed aseptically from C57BL/6J mice and the bone marrow cells were flushed out with alpha-modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 50 μ g/mL ascorbic acid and 2 mmol/L L-glutamine. A single-cell suspension was achieved by forcefully expelling the cells through a 22-gauge syringe needle. 1×10^7 total bone marrow cells were cultured in 28 cm² culture dishes in 5 mL of the above-mentioned medium in the absence or presence of 20 ng/mL epidermal growth factor (E9644, 0.2 mg; Sigma, St. Louis, MO, USA). After 24 hours, the non-adherent cells in the supernatants from the total bone marrow cells were transferred to another culture dish to form the first "pour-off"^[18], and the adherent cells were cultured further in fresh medium. The cells obtained by "pour-off" were used in two different ways, *i.e.*, for induction into neural cells and for methylene blue staining. This process was repeated daily for a total of five pour-offs. The medium was changed every 3 days. Each group of cells was cultured for up to 12 days, following which the cells were washed with PBS, fixed and stained with methylene blue (Sigma) for total colonies. The number of colonies per dish was determined manually and the positive colony area was measured by computer-assisted image analysis (Illustrator-CS2; Adobe Systems, San Jose, CA, USA).

Induced differentiation of total bone marrow cell-derived and non-adherent bone marrow cell-derived MSCs in vitro

Although a previous study reported that mouse bone marrow-derived MSCs can differentiate into neuronal-like cells^[36], it remains unknown whether non-adherent bone marrow cell-derived MSCs can differentiate into neuronal-like cells. To investigate this hypothesis, the "pour-off" bone marrow cell cultures were performed in the presence of 20 ng/mL epidermal growth factor (Sigma) as described above. After 10 days, the total bone

marrow cell-derived and the pour-off 1–4-derived MSCs were harvested by digesting with 0.25% trypsin/ 0.02% ethylenediamine tetraacetic acid. The cells derived from the pour-off 1–4 cultures were referred to as non-adherent bone marrow cell-derived MSCs. The total bone marrow cell-derived or non-adherent bone marrow cell-derived MSCs were plated at 2 000 cells/cm² in complete medium with the addition of 20 ng/mL basic fibroblast growth factor (Gibco), 20 ng/mL human epidermal growth factor (Gibco) and 10 ng/mL human nerve growth factor (Invitrogen, Carlsbad, CA, USA). The medium was changed every 3 days. After 14 days, the cultures were ended, washed with PBS, fixed and stained immunocytochemically for neurofilament-200 and NeuN as described below.

Establishment of a focal brain ischemia model in mice

The focal cerebral ischemic mouse model was established by the middle cerebral artery occlusion as described previously^[37]. Briefly, the wild type C57BL/6J mice were anesthetized with 2% chloral hydrate (0.1 mL/5 g) and a midline cervical incision was made to expose the right common carotid artery, which was then ligated, and a small opening was made in the common carotid artery 5 mm down from the bifurcation of the common carotid artery. A 5-0 nylon monofilament suture, with the tip rounded by heating, was introduced into the internal carotid artery through the opening in the stump and stopped when inserted about 11–13 mm from the bifurcation of the common carotid artery or a tight resistant was felt. After 60 minutes of occlusion, the nylon suture was removed to allow reperfusion. The mice that rotated to the left after recovery from anesthesia were regarded as successful models. Seven days after middle cerebral artery occlusion, these mice were used for the transplantation experiments with donor non-adherent bone marrow cell-derived MSCs.

Preparation and transplantation of non-adherent bone marrow cell-derived MSCs

The bone marrow cells were collected from the β -galactosidase transgenic mice. The "pour-off" bone marrow cell cultures were performed in the presence of 20 ng/mL epidermal growth factor as described above. The non-adherent bone marrow cell-derived MSCs were harvested from the pour-off 1–4 cultures and used as donor cells for transplantation. Seven days after middle cerebral artery occlusion, a 10 μ L Hamilton syringe containing semi-suspended non-adherent bone marrow-derived MSCs (1×10^6 in 3 μ L PBS) was inserted stereotaxically through the burr hole into the right stri-

tum (ischemic area) over a 10-minute period at coordinates of 2.0 mm lateral to the midline, 3.5 mm vertically, anterior-posterior at the zero point to the bregma^[38]. The needle was retained in the striatum for an additional 5-minute interval to avoid donor reflux. The mice in the control group were injected with 3 μ L PBS in the same way.

LacZ staining

At 8 weeks after transplantation, the mice were perfused with ice-cold saline, and the brain was removed and fixed with periodate-lysine-paraformaldehyde stationary liquid fixative^[39] overnight at 5°C. The pre-embedding LacZ staining was performed as described previously^[40]. Briefly, the fixed samples were washed three times for 30 minutes in LacZ wash buffer (PBS containing 2 mmol/L MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, pH 7.3) and incubated in staining solution (0.5 mg/mL X-gal, 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferricyanide in LacZ wash buffer) at 37°C overnight with shaking and protection from light. After another fixation for 24 hours, samples were dehydrated in graded alcohols and embedded in low-melting-point paraffin, after which 5 μ m sections were cut on a rotary microtome and then de-waxed, mounted with balsam neutral, and observed with a light microscope (Leica, Solms, Germany).

Immunocytochemical/immunohistochemical staining

Cultured cells in culture dishes or paraffin sections of brains were stained using the avidin-biotin-peroxidase complex technique. The cultured cells or dewaxed sections of brain were first treated with 10% normal goat serum in PBS containing 0.5% bovine serum albumin for 30 minutes at room temperature to block non-specific binding. Primary monoclonal rat anti-mouse antibody [β -galactosidase (1:400; Promega, Madison, WI, USA, Z3783), neurofilament-200 (1:500; Sigma) or NeuN (1:500; Chemicon, Santa Cruz, CA, USA, MAB377)] was applied to cells or tissues overnight at room temperature. As a negative control, the preimmune serum or Tris-buffered saline (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.01% Tween-20, pH 7.6) was substituted for the primary antibody. After washing with high salt buffer (50 mmol/L Tris-HCl, 2.5% NaCl, and 0.05% Tween-20, pH 7.6) for 10 minutes at room temperature followed by two 10-minute washes with Tris-buffered saline, the cells were incubated with secondary antibody [biotinylated goat anti-rat IgG, (1:200; Sigma)]. Cells or sections were then washed as before and incubated with the Vectastain Elite avidin-biotin-peroxidase complex kit (Vector Labor-

atories, Inc., Ontario, Canada) for 45 minutes. After washing as before, brown pigmentation to demarcate regions of immunostaining was produced by using a 3,3'-diaminobenzidine kit (SK-4100, Vector Laboratories, Inc.). After washing with distilled water, the cells and sections were counterstained with hematoxylin and mounted with balsam neutral. For the double staining of β -galactosidase and NeuN, following immunostaining for β -galactosidase (brown color localized in cytoplasm), another immunostaining procedure for NeuN (gray color localized in nuclei) was performed on the same sections. For the second immunostaining procedures, gray pigmentation was produced using a Vector SG kit (Vector Laboratories, Inc.) and observed by light microscopy.

Statistical analysis

Data are presented as mean \pm SEM. Statistical comparisons were made by one-way analysis of variance using SPSS 18.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

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