

Bone Marrow-Derived Stem Cells Can Differentiate into Retinal Cells in Injured Rat Retina

MINORU TOMITA,^{a,b} YASUSHI ADACHI,^{a,c,d} HARUHIKO YAMADA,^b KANJI TAKAHASHI,^b
KATSUJI KIUCHI,^{b,e} HARUKI OYAIZU,^a KAZUYA IKEBUKURO,^a HIROYUKI KANEDA,^a
MIYO MATSUMURA,^{b,c,d} SUSUMU IKEHARA^{a,c,d}

^aFirst Department of Pathology, ^bDepartment of Ophthalmology, ^cTransplantation Center, ^dRegeneration Research Center for Intractable Disease, and ^eSecond Department of Pathology, Kansai Medical University, Osaka, Japan

Key Words. *Retinal transplantation · BMT · BMSCs*

ABSTRACT

It has recently been shown that bone marrow cells can differentiate into various lineage cells including neural cells *in vitro* and *in vivo*. We therefore examined whether bone marrow stem cells can differentiate into retinal neural cells in adult rats. PKH-67-labeled stem cell-enriched bone marrow cells (BMCs) were injected into the vitreous space of eyes in which the retinas had been mechanically injured using a hooked needle. Two weeks after the injection of these cells, immunohistochemical examinations were carried out. The stem cell-enriched

BMCs had been incorporated and had differentiated into retinal neural cells in the injured retina. The stem cell-enriched BMCs had accumulated mainly in the outer nuclear layer around the injured sites. The incorporated cells expressed glial fibrillary acidic protein, calbindin, rhodopsin, and vimentin. These results raise the possibility that stem cell-enriched BMCs have the ability to differentiate into retinal neural cells, and that the injection of stem cell-enriched BMCs into the retina would help repair damaged retinal cells. *Stem Cells* 2002;20:279-283

INTRODUCTION

Recently, it has been reported that bone marrow cells (BMCs) differentiate into various cells, including hepatocytes [1], endothelial cells of the blood vessel [2], epithelial cells of the stomach, esophagus, small intestine, large intestine and bronchus [3], cardiac muscle [4], and skeletal muscle [5]. It has also been reported that BMCs differentiate into neural cells and astrocytes *in vitro* [6, 7] and also into astrocytes *in vivo* when transplanted into the normal [8, 9] or ischemic brain [10]. Moreover, the intravenous injection

of BMCs into mice has been shown to induce neuronal differentiation in the brain [11-13]; BMCs have the capacity to differentiate into myelin-forming cells *in vivo* and to repair demyelinated spinal cord axons [14]. These findings suggest that BMCs can differentiate into nerve cells under the appropriate conditions. On the other hand, neural stem cells can generate hematopoietic cells *in vivo*, indicating that neural precursor cells are not restricted to the differentiation of neural-lineage cells [15]. There are many studies of retinal transplantation using the newborn or the embryonic

Correspondence: Susumu Ikehara, M.D., First Department of Pathology, Kansai Medical University, Fumizono-cho, Moriguchi City, Osaka, Japan, 570-8506. Telephone: 81-6-6992-1001, ext 2474 or 2475; Fax: 81-6-6992-1219; e-mail: ikehara@takii.kmu.ac.jp Received November 26, 2001; accepted for publication March 22, 2002. ©AlphaMed Press 1066-5099/2002/\$5.00/0

brain [16, 17] and retina-derived neural cells [18-21]. Moreover, hippocampus-derived neural stem cells have been shown to differentiate into neural-forming cells, which are then incorporated into the injured retina [22-25]. However, it is still unclear whether BMCs can differentiate into nerve cells in the retina. The present study demonstrates that BMCs can differentiate into retinal neural cells (cells of the outer nuclear layer [ONL]) *in vivo*, and that the cells express glial fibrillary acidic protein (GFAP), calbindin, rhodopsin, and vimentin.

MATERIALS AND METHODS

Preparation of Stem Cell-Enriched BMCs for Grafting

We prepared partially purified stem cell-enriched BMCs as previously described [26-28]. Briefly, 5-fluorouracil (Kyowa Hakko Kogyo; Tokyo, Japan) was injected into the peritoneal cavity of 4-week-old male Brown Norway rats (Clea Japan; Osaka, Japan) at 150 mg/kg body weight 48 hours before sacrifice. The BMCs were obtained from femurs and tibias, and were fractionated by density centrifugation using Lymphoprep™ density solution (density, 1.077; Nycomed Pharma; Oslo, Norway; <http://www.nycomed.com>). After centrifugation, whole mononuclear cells, as stem cell-enriched BMCs, were collected from the interface and were labeled using PKH67 (green fluorescence; MINI-67; Sigma; Saint Louis, MO; <http://www.sigmaaldrich.com>).

Rats and Grafting Procedure

Rats (15- to 18-week-old Brown Norway rats; $n = 6$) were anesthetized with pentobarbital sodium (10 mg/kg body weight; Somnopentyl; Pitman-Moore; Washington Crossing, NJ), which was injected into the abdominal cavity. The pupils were dilated with 0.5% tropicamide (Santen; Osaka, Japan; <http://www.santeninc.com>). The corneas were further anesthetized with drops of 0.4% oxybuprocaine hydrochloride (Santen). The eyeballs were perforated at the equator with a 30-gauge needle, and the retina was injured by scratching parallel to the equator between the retinal vessels under direct observation with a surgical microscope equipped with a plano-concave contact lens for rats (Kyocon; Kyoto, Japan). Special care was taken to injure the whole layer of the retina. After the injury, 20 μ l of the cell suspension, containing 200,000 cells, were slowly injected into the intravitreal space with the injector (six rats, six eyes). As a control, 20 μ l of normal saline solution were injected into the intravitreal space of the injured eyes (six rats, six eyes). The results from one eye of the control group were excluded due to complication of massive vitreous hemorrhage.

Tissue Sectioning

The animals were sacrificed 2 weeks after injection. The eyes were enucleated and embedded in optimal cutting temperature compound (Miles; Elkhart, IN) after adjustment of their horizontal planes parallel to the cutting plane, and 2 μ m frozen sections were made in a cryostat. Continuous sections including the injury site were cut for each eye.

Antibodies

Antibodies (Abs) used in this study were as follows: rabbit polyclonal anti-GFAP; 1:1,000; Sigma), rabbit antirhodopsin (1:500; LSL; Tokyo, Japan), mouse monoclonal anticalbindin-D28K (1:1,000; Sigma), monoclonal mouse antivimentin (1:100; Dako; Glostrup, Denmark; <http://www.dako.dk>), anti-rat CD45 (1:50; PharMingen; San Diego, CA; <http://www.bdbiosciences.com/pharmingen>), R-phycoerythrin (PE)-conjugated goat anti-mouse Ab (1:50; PharMingen), and R-PE-conjugated goat anti-rabbit Ab (1:500; Southern Biotechnology Associates; Birmingham, AL; <http://www.southernbiotech.com>).

Immunocytochemistry

The specimens, which had been fixed with acetone, were stained with rabbit Abs (anti-GFAP or antirhodopsin) or mouse Abs (anticalbindin and antivimentin) followed by staining with PE-labeled goat anti-rabbit Ab or PE-labeled goat anti-mouse Ab. For staining with anti-CD45, the specimens were stained with PE-labeled anti-CD45 Ab. The stained specimens were observed using a confocal microscope (Fluoview; Olympus; Tokyo, Japan; <http://www.olympus.com>).

RESULTS

Incorporation and Distribution of Injected Cells

Since it has been reported that BMCs have the ability to differentiate into various lineage cells, we examined whether they can differentiate into retinal cells. Previously, *Nishida et al.* reported that hippocampus-derived neural stem cells could not migrate into the intact retina, whereas they could do so into the injured retina [22]. Therefore, we injected BMCs into the vitreous space after injuring the retina. At first, we injected whole BMCs into the vitreous space after injuring the retina, but this maneuver induced severe inflammation in the eye. Therefore, we enriched bone marrow stem cells, as described above, and injected these into the vitreous space after injuring the retina. Two weeks after transplantation, we killed the injected rats and examined their eyes. The injected stem cell-enriched BMCs had been incorporated into the host retina in 83% of the experimental eyes (5/6). Injured retina, after injection of

saline, showed rolling in the retina (Fig. 1A), while injured retina, after injection of stem cell-enriched BMCs, showed cell proliferation in the ONL (Fig. 1B). These findings suggest that mechanical injury induces only disorders of the retina, but the injection of stem cell-enriched BMCs induces proliferation of the retinal neural cells.

Next, we examined whether the increased retinal neural cells were derived from injected BMCs or from host-derived retinal cells due to *de novo* proliferation under the stimulation of injected BMCs. The grafted cells, which expressed green fluorescence, were distributed around the injured sites (Figs. 1C and D) where the layers of the retina had been disordered. The patterns of distribution of the grafted cells were similar in all five experiments. Grafted cells were observed not only at the site of injury, where the normal retinal structure had been destroyed, but also in the surrounding area, where the normal retinal structure had been retained. Most of the grafted cells were located in the ONL, but some were in the inner nuclear layer (INL). When sections were stained with HE, the grafted cells were seen to be morphologically similar to the cells in the ONL. These results suggest that the injected stem cell-enriched BMCs had migrated into the retina,

followed by proliferation and differentiation into retinal neural cells (Figs. 1B and D).

Immunohistochemistry on Sections after Transplantation

To clarify that stem cell-enriched BMCs inoculated into the eyes have the ability to differentiate into nerve cells in the eye, immunohistochemical studies using confocal microscopy were performed 2 weeks after transplantation. The cell-type markers used were GFAP for astrocytes and Müller cells, calbindin for horizontal and amacrine cells, vimentin for glial cells, rhodopsin for rod photoreceptor cells, and CD45 for hematopoietic cells. The injected cells, which had been labeled with PKH-67, expressed green fluorescence, while the expressions of GFAP, carbindin, vimentin, and rhodopsin, which were labeled with R-PE-labeled second Abs, were detected as red fluorescence. Therefore, the incorporated bone marrow-derived cells that expressed GFAP, carbindin, vimentin, or rhodopsin expressed yellow fluorescence. As shown in Figure 2, some of the injected cells expressed GFAP, carbindin, vimentin, or rhodopsin (arrows), while some expressed green fluorescence, suggesting that some of the incorporated bone marrow cells had differentiated into retinal cells. We also examined the

Figure 1. Bone marrow cells (BMCs) migrate into the injured retina. Bone marrow stem cells were partially purified, followed by labeling with PKH-67. Stem cell-enriched BMCs or saline were then injected into the vitreous space after injury of the retina. Two weeks after injection, the eyes were examined. (A) Only saline was injected into the injured eyes as a control. The outer nuclear layer (ONL) rolled, but cells in the ONL did not proliferate (arrow) (original magnification $\times 50$, HE staining). (B) Cells in the ONL proliferated when stem cell-enriched BMCs were injected (arrows) (original magnification $\times 50$, HE staining). (C) The distribution of injected stem cell-enriched BMCs in the injured retina is observed as the cells showing green fluorescence (arrows), using confocal microscopy (original magnification $\times 20$). (D) A continuous section of (C) was prepared, followed by staining with HE (original magnification $\times 25$), and the figure shows the same place as (C). Proliferated cells in the ONL correspond to green fluorescence in (C) (arrows). Representative data from five independent experiments are shown.

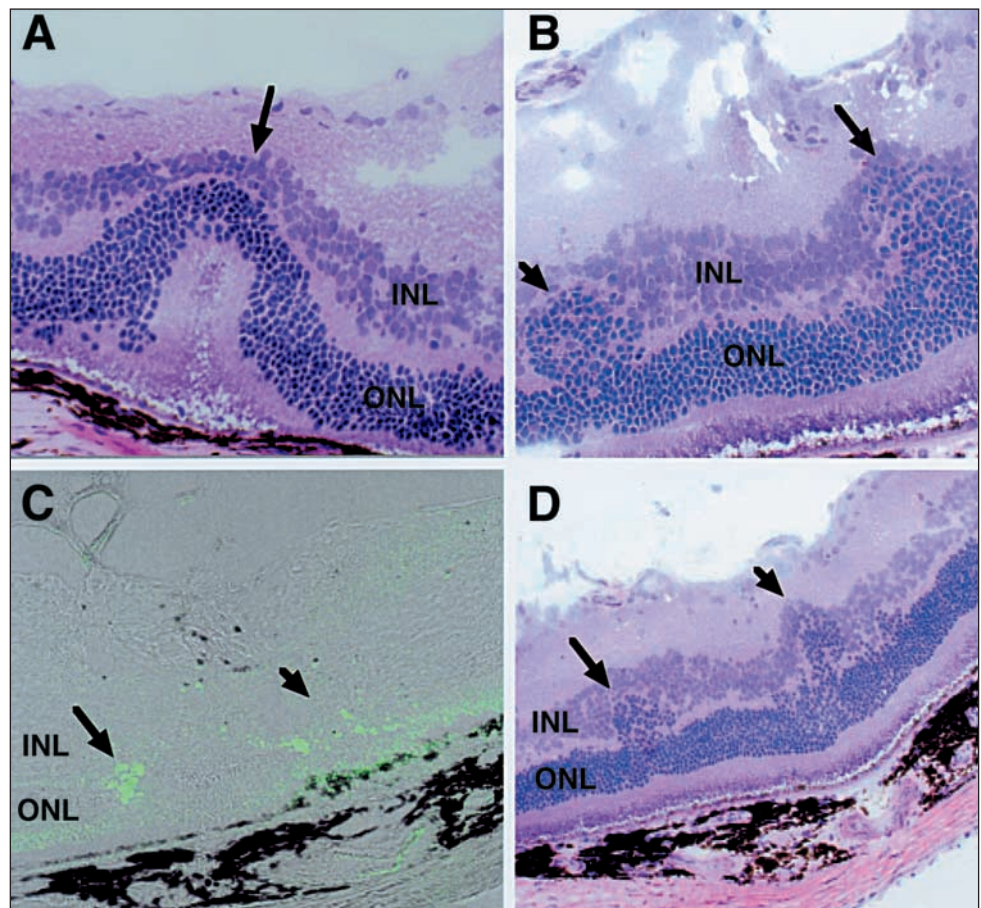
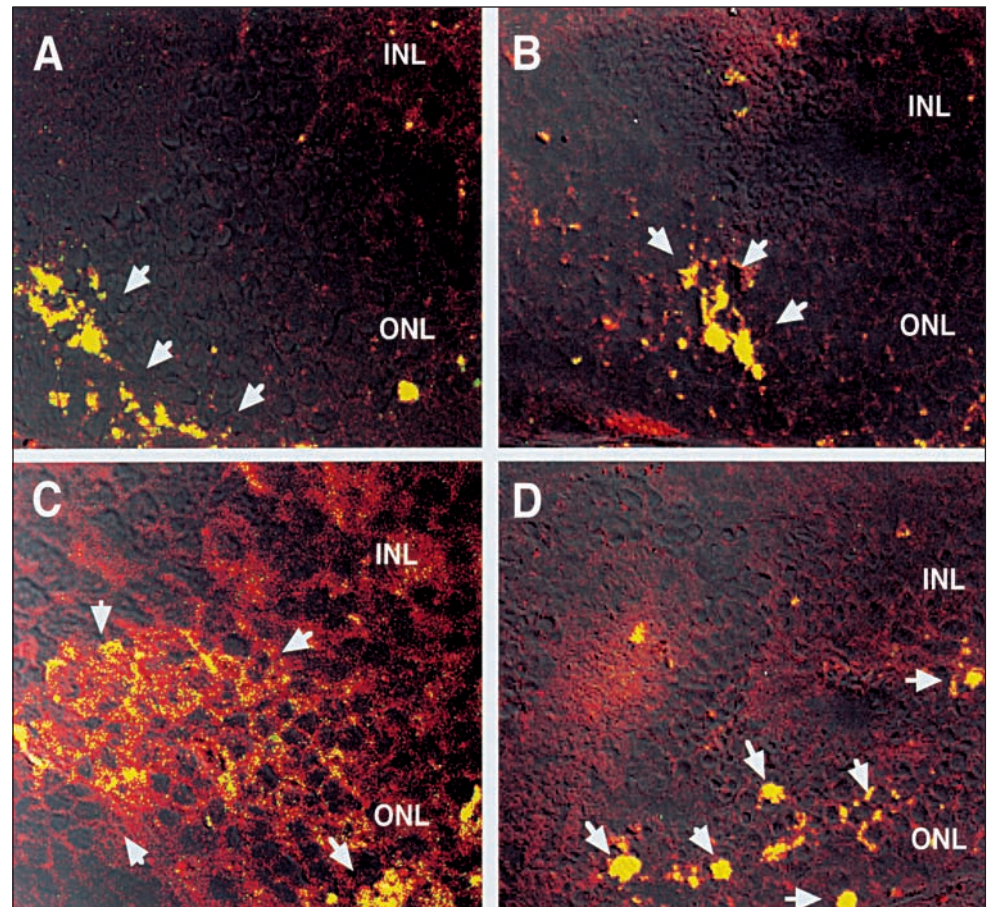


Figure 2. Expression of glial fibrillary acid protein (GFAP) (A), calbindin (B), vimentin (C), and rhodopsin (D) in the injected cells. Injected cells, which had been labeled with PKH-67, emitted green fluorescence, while expressions of GFAP, calbindin, vimentin, and rhodopsin were detected with red fluorescence. After immunohistochemical staining, injected cells that express GFAP (A), calbindin (B), vimentin (C), and rhodopsin (D) express yellow fluorescence (arrows), while injected cells that do not express the neuron-specific or retina-specific markers express green fluorescence. The cells expressing yellow fluorescence are shown. The original magnification of (A), (B), (C), and (D) was $\times 60$. Representative data from five independent experiments are shown.



expression of CD45 to detect hematopoietic cells. We detected CD45⁺ cells in the retina injected with stem cell-enriched BMCs, but CD45⁻ cells were more dominant than CD45⁺ cells in the retina (data not shown), and stem cell-enriched BMCs did not express GFAP, calbindin, vimentin, or rhodopsin (data not shown). These results strongly suggest that the injected stem cell-enriched BMCs differentiated into the retinal neural cells.

DISCUSSION

It has been reported that BMCs differentiate into neural cells and astrocytes in cell culture [6, 7]. In this report, we have shown that stem cell-enriched BMCs injected into injured eyes can differentiate into retinal neural cells *in vivo*.

There have been several transplantation studies examining the differentiation of BMCs into neural retinal cells. Radner *et al.* subretinally transplanted the neural retinal tissue of newborn normal mice into C3H/HeJ (*rd/rd*) mice. The transplanted cells were incorporated into the retina and resided between the INL and the retinal pigment epithelium [16]. When brain-derived cell lines were transplanted into the retina, the cells were integrated into all major retinal cell layers [17]. Putative progenitors of retinal cells contained in the embryonic retina survived and differentiated into retinal cells in the transplanted eyes [18]. Embryonal retinas transplanted into the subretinal space survived in the host eyes [19-21]. In the above reports, retinal tissue was

transplanted, and it is, therefore, difficult to apply these techniques to autotransplantation in clinical treatment. It has also been shown that hippocampus-derived neural stem cells, which have the capacity to differentiate into neural cells, astrocytes, and oligodendrocytes, migrate into the injured retina and differentiate into neural cells [22-25]; the incorporated cells express Map2ab, Map5 (the marker for mature neural cells), and GFAP, but not specific retinal cell markers (calbindin or rhodopsin) [22, 23]. In the present study, transplanted BMCs expressed not only GFAP and vimentin but also calbindin and rhodopsin, which are considered to be retinal cell-specific markers. It has been described that BMCs include pluripotent stem cells, which can differentiate into not only hematopoietic cells but also epithelial cells, endothelial cells, and nerve cells in the various organs. Therefore, our results suggest that BMCs contain more primitive stem cells than hippocampus-derived neural stem cells, and that the injection of BMCs into the eye can potentially rescue injured retinal tissue, even in humans.

ACKNOWLEDGMENT

We thank Ms. Tokuyama for her expert technical assistance and also Ms. Ando for the preparation of this manuscript.

REFERENCES

- 1 Lagasse E, Connors H, Al-Dhalimy M et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 2000;6:1229-1234.
- 2 Shi Q, Rafii S, Wu MH-D et al. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998;92:362-367.
- 3 Krause DS, Theise ND, Collector MI et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001;105:369-377.
- 4 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001;410:701-705.
- 5 Ferrari G, Cusella-De Angelis G, Coletta M et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 1998;279:1528-1530.
- 6 Woodbury D, Schwarz EJ, Prockop DJ et al. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000;61:364-370.
- 7 Sanchez-Ramos J, Song S, Cardozo-Pelaez F et al. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* 2000;164:247-256.
- 8 Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci USA* 1999;96:10711-10716.
- 9 Azizi SA, Stokes D, Augelli BJ et al. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. *Proc Natl Acad Sci USA* 1998;95:3908-3913.
- 10 Eglitis MA, Dawson D, Park K-W et al. Targeting of marrow-derived astrocytes to the ischemic brain. *Neuroreport* 1999;10:1289-1292.
- 11 Brazelton TR, Rossi FMV, Keshet GI et al. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 2000;290:1775-1779.
- 12 Mezey E, Chandross KJ, Harta G et al. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 2000;290:1779-1782.
- 13 Eglitis MA, Mezey E. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci USA* 1997;94:4080-4085.
- 14 Sasaki M, Honmou O, Akiyama Y et al. Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. *Glia* 2001;35:26-34.
- 15 Bjornson CRR, Rietze RL, Reynolds BA et al. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* 1999;283:534-537.
- 16 Radner W, Sadda SR, Humayun MS et al. Light-driven retinal ganglion cell responses in blind *rd* mice after neural retinal transplantation. *Invest Ophthalmol Vis Sci* 2001;42:1057-1065.
- 17 Warfvinge K, Kamme C, Englund U et al. Retinal integration of grafts of brain-derived precursor cell lines implanted subretinally into adult, normal rats. *Exp Neurol* 2001;169:1-12.
- 18 Chacko DM, Rogers JA, Turner JE et al. Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat. *Biochem Biophys Res Commun* 2000;268:842-846.
- 19 Ghosh F, Johansson K, Ehinger B. Long-term full-thickness embryonic rabbit retinal transplants. *Invest Ophthalmol Vis Sci* 1999;40:133-142.
- 20 Gjörloff K, Bruun A, Ghosh F. Cone photoreceptors in laminated retinal transplants. *Acta Ophthalmol Scand* 2001;79:366-369.
- 21 Ghosh F, Bruun A, Ehinger B. Immunohistochemical markers in full-thickness embryonic rabbit retinal transplants. *Ophthalmic Res* 1999;31:5-15.
- 22 Nishida A, Takahashi M, Tanihara H et al. Incorporation and differentiation of hippocampus-derived neural stem cells transplanted in injured adult rat retina. *Invest Ophthalmol Vis Sci* 2000;41:4268-4274.
- 23 Takahashi M, Palmer TD, Takahashi J et al. Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. *Mol Cell Neurosci* 1998;12:340-348.
- 24 Palmer TD, Takahashi J, Gage FH. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* 1997;8:389-404.
- 25 Kurimoto Y, Shibuki H, Kaneko Y et al. Transplantation of adult rat hippocampus-derived neural stem cells into retina injured by transient ischemia. *Neurosci Lett* 2001;306:57-60.
- 26 Doi H, Inaba M, Yamamoto Y et al. Pluripotent hemopoietic stem cells are c-kit^{low}. *Proc Natl Acad Sci USA* 1997;94:2513-2517.
- 27 Tajima F, Deguchi T, Laver JH et al. Reciprocal expression of CD38 and CD34 by adult murine hematopoietic stem cells. *Blood* 2001;97:2618-2624.
- 28 Sugiura K, Hisha H, Ishikawa J et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro. *STEM CELLS* 2001;19:46-58.