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Bone marrow stromal cell transplantation enhances recovery of motor function after lacunar stroke in rats

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This study was aimed to clarify if the bone marrow stromal cells (BMSCs) significantly improve functional outcome after lacunar stroke when stereotactically transplanted into the brain. Ouabain, a Na/K ATPase pump inhibitor, was stereotactically injected into the right striatum of Wistar rats. One week later, the superparamagnetic iron oxide (SPIO)-labeled rat BMSCs (n=7) or vehicle (n=8) were stereotactically transplanted into the left striatum. Using rotarod test, motor function was serially evaluated through the experiment. A 7.0-T MR apparatus was employed to serially monitor the migration of BMSCs in the host brain. Histological analysis was performed at 7 weeks after ouabain injection, i.e., 6 weeks after BMSC transplantation. Ouabain injection yielded the reproducible, focal lesion in the right striatum, causing continuous motor dysfunction throughout the experiment. BMSC transplantation significantly enhanced the recovery of motor function after ouabain injection. MR imaging demonstrated that the BMSCs aggressively migrated towards the lesion through the corpus callosum. Histological analysis supported the findings on MRI. The BMSCs significantly enhanced the neurogenesis in the subventricular zone (SVZ) on both sides. Some of them also expressed neuronal or astrocytic phenotypes in the neocortex, SVZ, corpus callosum, and peri-lesion area. These findings strongly suggest that the BMSCs may serve therapeutic impacts on lacunar stroke when stereotactically transplanted at clinically relevant timing.

Key words: bone marrow stromal cell, lacunar stroke, transplantation, MRI

INTRODUCTION

Stroke is still a leading cause of death and disability, but few treatment options exist despite intensive research (Savitz and Fisher 2007). Lacunar infarction is a subtype of ischemic stroke that results from occlusion of one of the penetrating arteries that provides blood to the deep brain structures. It is estimated that lacunar infarction may account for at least 25% of all ischemic strokes with an annual incidence of approximately 15 per 100 000 people (Sacco et al. 2006). The patients with lacunar infarction are known to have a better survival than those with other subtypes of ischemic stroke, but their neurological deficits, including pure motor hemiparesis and dysarthria-clumsy hand syndrome, often remain as sequelae.

Recently, the therapeutic potential of cell transplantation has been studied in various pathological conditions of the central nervous system (CNS), because the damaged CNS has the limited regenerative capacity (Jablonska and Lukomska 2011). Especially, growing evidence suggests that bone marrow stromal cells (BMSCs) hold a great potential as cell source of cell therapy for ischemic stroke. Thus, the BMSCs can survive in the infarct brain, migrate towards cerebral infarct, express neural phenotypes, and promote functional recovery, when transplanted into various kinds of animal model of middle cerebral artery occlusion (Abe et al. 2012, Drela et al. 2013, Kuroda 2013). However, it is still obscure whether BMSC transplantation could be effective against lacunar stroke. Based on these observations, therefore, this study was aimed

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to clarify the therapeutic effects of BMSC transplantation on lacunar stroke model of rats.

METHODS

Preparation of rat BMSCs

All animal experiments were approved by the Animal Studies Ethical Committee at Hokkaido University Graduate School of Medicine. Green fluorescence protein (GFP)-transgenic rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The BMSC were isolated from 8-week-old GFP rats as described previously (Ito et al. 2011, Sugiyama et al. 2011a, Kawabori et al. 2012, 2013, Miyamoto et al. 2013). The cells were passed three times for subsequent experiments. Prior to transplantation, the BMSCs were magnetically labeled with a superparamagnetic iron oxide (SPIO), 1-µl/ml Resovist (Ferucarbotran, 27.9 µg Fe/ml, Fujifilm RI Pharma Co., Ltd., Tokyo, Japan) for 24 h, as described previously (Ito et al. 2011, Shichinohe et al. 2012). The labeled BMSCs were trypsinized and resuspended in PBS.

Rat lacunar infarct model and BMSC transplantation

Male Wistar rats weighing 240 to 260 g (n=15) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Lacunar lesion was induced by stereotactically injecting ouabain, a Na/K ATPase pump inhibitor, into the right striatum, as described by Janowski and others (2008) with minor modifications (Janowski et al. 2008). Anesthesia was induced by inhalation of 4.0% isoflurane in N_2O/O_2 (70:30) and maintained with 1.5% isoflurane in N_2O/O_2 (70:30). The rat was fixed to a stereotactic apparatus (Model DKI-900, David Kopf Instruments, Tujunga, CA). A burr hole was made 3 mm right to the bregma, using a small dental drill. A Hamilton syringe was inserted 5 mm into the brain parenchyma from the surface of dura mater, and 1 µl of 2.5-mM ouabain (Sigma, St. Louis, MO) was injected over 5 min, using an automatic microinjection pump (Model KDS-310, Muromachi Kikai Co., LTD., Tokyo, Japan). After the injection, the needle was left *in situ* for 5 min to avoid leakage of the injected fluid through the needle tract. Core temperature was main-

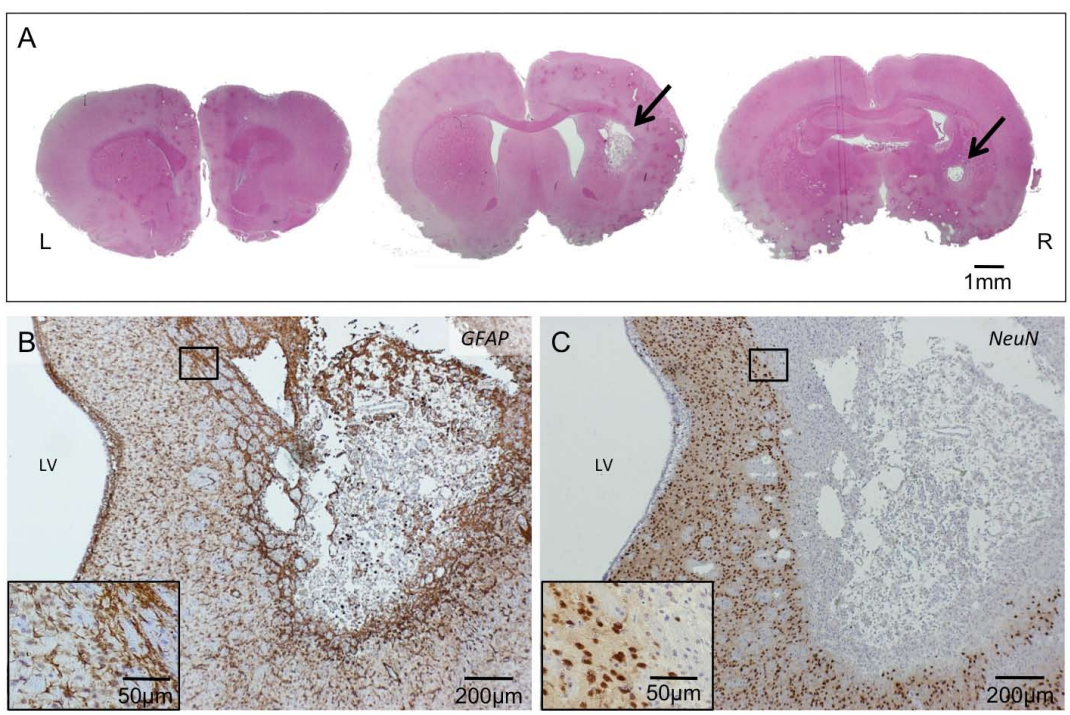

Fig. 1. (A) HE staining demonstrates that the ouabain-induced lesion is located in the right striatum (arrows). Photomicrographs of immunohistochemistry against GFAP (B) and NeuN (C) show the proliferation of reactive astrocytes and selective loss of neurons around the ouabain-induced lesion. The insets in Panels B and C are the magnified images, which are located on the square on each panel. (LV) lateral ventricle.

tained between 36.5 and 37.5°C during the procedures.

Seven days after the insult, the BMSC were stereotactically transplanted into the left striatum, as described before (Ito et al. 2011, Sugiyama et al. 2011a, Kawabori et al. 2012, 2013, Miyamoto et al. 2013). Briefly, the BMSCs were suspended in phosphate-buffered solution (PBS). The animals were anesthetized as mentioned above, and a burr hole was made 3 mm left to the bregma, using a small dental drill. A Hamilton syringe was inserted 4 mm into the brain parenchyma. Then, 10 µl of BMSC suspension (5×10^5 cells, n=7) or vehicle (PBS, n=8) was injected into the left striatum over 5 min, using an automatic microinjection pump. All animals in both groups were treated subcutaneously with 10-mg/kg cyclosporine A daily until their sacrifice.

Assessment of motor function

Motor function of the animals was semi-quantitatively assessed before and after ouabain injection, using a rotarod treadmill (Model MK-630, Muromachi Kikai Co., Tokyo, Japan). The rotarod was set to the acceleration mode of 4 to 40 revolutions per min for 3 min, as previously described (Ito et al. 2011, Sugiyama et al. 2011b, Kawabori et al. 2012, 2013, Miyamoto et al. 2013). In this study, all animals were randomly divided into two groups and the investigators were blinded to the grouping of animals during experiments.

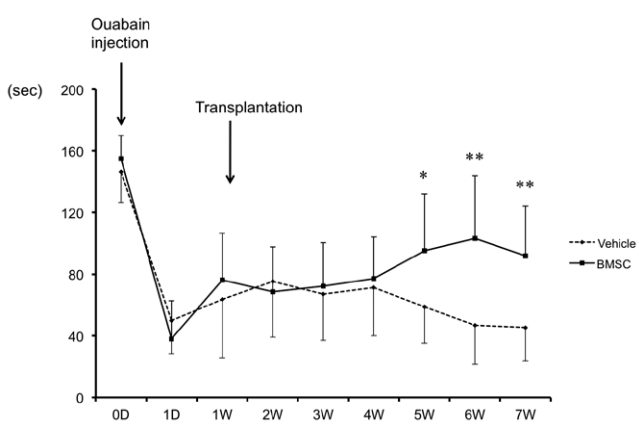

Fig. 2. Rotarod test. Line graph shows the temporal profile of functional recovery after ouabain injection in vehicle- (dotted line) and BMSC-treated animals (solid line). *P<0.05, **P<0.01.

In vivo serial MR tracking of transplanted BMSCs

Using an MRI apparatus, the behaviors of transplanted BMSCs were serially monitored at 2, 8, 14, 28, and 49 days after ouabain injection (BMSC group: n=4). All MR imaging was acquired using a small animal, horizontal bore, 7.0-Tesla MR scanner (Unity INOVA, Varian, Inc., Palo Alto, CA) interfaced to a VNMR console (Varian Inc.), as described previously (Ito et al. 2011). Briefly, the animals were anesthetized in the above-mentioned condition and were placed on nonmagnetic holder equipped with a nose cone for administration of anesthetic gas and to minimize error in the repeatability of positioning of the head of rats. During the imaging procedure, core temperature was kept between 36.5°C and 37.5°C, using feedback-controlled water bath. To detect the SPIO-labeled BMSCs in the brain, coronal T2-weighted images were acquired using standard two-dimensional Fourier transform, multislice (nine slices, 1 mm thick) and spin echo T2 sequence. The sequence parameters were as follow: repetition time (TR) = 2 500 ms, echo time (TE) = 60 ms, number of acquisitions (AC) = 4 times, field of view (FOV) = 30 × 30 mm, and matrix = 512 × 512 (Ito et al. 2011).

Histological analysis

At 7 weeks after ouabain injection, the animals deeply anesthetized with 4.0% isoflurane in N_2O:O_2 (70:30) and were transcardially perfused with 20 ml of heparinized saline, followed by 50 ml of 4% paraformaldehyde. The brain tissue was removed and embedded in paraffin. Then, 4-µm-thick coronal sections were prepared for subsequent analysis. To histologically identify the ouabain-induced lesion, the sections were stained with hematoxylin and eosin (HE). To evaluate the distribution of SPIO-labeled cells, the sections were stained with Turnbull blue method, as described previously (Yano et al. 2005, Ito et al. 2011).

Immunohistochemistry was performed to precisely evaluate the ouabain-induced lesion. Briefly, the deparaffinized sections were processed through antigen retrieve for 2 min by pressure pot. The sections were treated with primary antibody against NeuN (mouse monoclonal, 1:400 dilution, Chemicon, CA), glial fibrillary acidic protein (GFAP; mouse monoclonal, 1:100, BD Biosciences) at 4°C over night. Then, DAKO EnVision+Kit

and DAB Substitute Kit (DAKO Cytomation, Kyoto, Japan) were applied according to their instructions, and hematoxylin was used for counterstaining.

To assess the neurogenesis in the host brain and the fate of transplanted BMSCs, double fluorescence immunohistochemistry was also performed. The sections were treated with primary antibody against NeuN (1:400, Chemicon), GFAP (1:100, BD Biosciences) or doublecortin (DCX; mouse monoclonal, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C over night. They were treated with Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR) at 4°C for 4 h. Subsequently, they were incubated with the antibody against GFP (mouse monoclonal, 1:100, Santa Cruz) tagged with a fluorescence label (Zenon Alexa Fluor 488 Mouse IgG Labeling Kit, Molecular Probes Inc.) at room temperature for 1 h. All sections were counterstained with DAPI (Sigma). The fluorescence emitted was observed through each appropriate filter on fluorescence microscope (BX51, Olympus, Tokyo, Japan) and was digitally photographed using a cooled CCD camera (model VB-6000/6010, Keyence, Osaka, Japan).

The number of DCX-positive cells in the subventricular zone (SVZ) was counted by setting the regions of interest (ROIs; 400 μm × 320 μm) on both the ipsi- and contralateral sides in each animal. The software, Image J (Ver. 1.32J, National Institutes of Health, Bethesda, MD), was employed to count their numbers.

Statistical analysis

All data are expressed as mean ± SD. The data were compared using the paired or unpaired *t*-test for cases involving two groups as appropriate. Values of $P<0.05$ were considered statistically significant.

RESULTS

Ouabain-induced lesion

All experimental animals survived through the experiment. On HE staining, the lesions were limited in right striatum at 7 weeks after ouabain injection. Some animals had ventricular dilatation in the ipsilateral hemisphere. Immunohistochemistry against NeuN and GFAP revealed that necrotic core lesion was surrounded by the marginal area with selective neuronal damage and reactive gliosis (Fig. 1). There were no

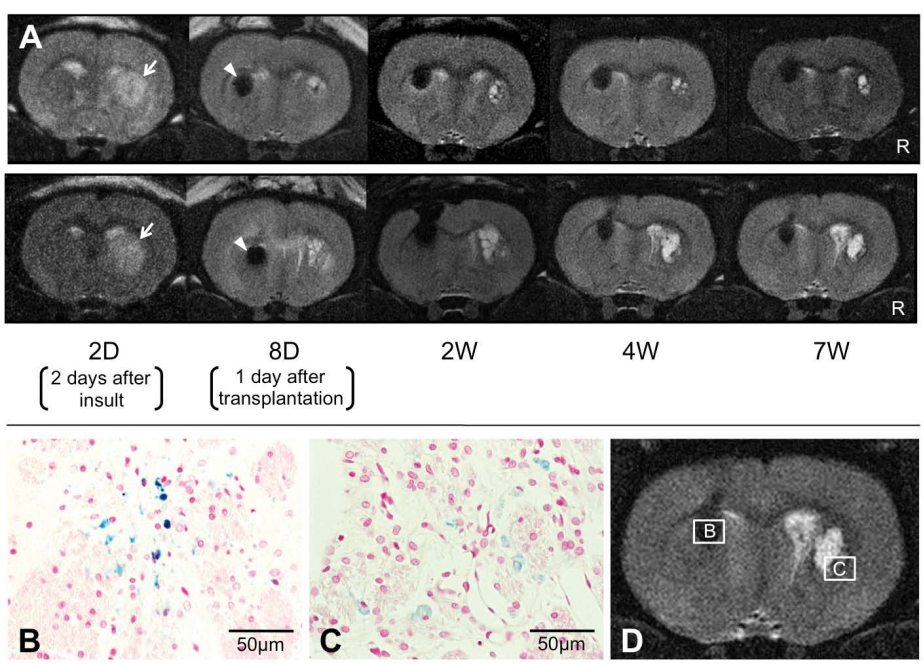

Fig. 3. (A) Serial T2-weighted MR images of 2 representative animals that are treated with the SPIO-labeled BMSCs. Arrows indicate the ouabain-induced lesion in the right striatum. Arrowheads indicate the signal loss area after stereotactic transplantation of the SPIO-labeled BMSC into the left striatum. Photomicrographs of Turnbull blue staining at the transplantation site (B) and the peri-lesion area (C). The Boxes B and C in panel D indicate the location of Panels B and C, respectively.

significant differences between the BMSC- and vehicle-treated animals.

Effects of BMSC transplantation on motor function

All the animals showed mild weakness of the left extremities immediately after ouabain injection. The duration time the animals could stay on rotarod dramatically decreased at one and 7 days after ouabain injection. The BMSCs or vehicle was transplanted at 7 days after ouabain injection. Both BMSC- and vehicle-treated animals showed no significant recovery of motor function during 4 weeks after ouabain injection. At 5 weeks after ouabain injection, however, motor function started to recover in the BMSC-treated animals. As shown in Figure 2, BMSC transplantation significantly enhanced the recovery of motor function at 5, 6, and 7 weeks after ouabain injection ($P<0.05$, $P<0.01$, and $P<0.01$, respectively).

MRI

MR imaging could serially monitor the ouabain-induced lesion in the right striatum. Thus, T2-weighted images clearly showed the lesion in the right striatum as a high-intensity area at 2 days after ouabain injection. The lesion gradually decreased in size thereafter. Gradual dilatation of the right lateral ventricle was simultaneously observed (Fig. 3).

MR imaging could also visualize the behaviors of transplanted BMSCs. At one day after transplantation, the SPIO-labeled cells were clearly delineated in the left striatum, i.e., the injection site as a strong signal loss. Subsequently, they started to migrate through the corpus callosum and reach the ouabain-induced lesion within 2 weeks post-transplantation (Fig. 3). Turnbull blue staining revealed that the SPIO- positive cells were distributed in the borderzone area around ouabain-induced lesion as well as in the injection site, which correlated very well with the findings on final MRI (Fig. 3).

Histological analysis

In the BMSC-treated animals, immunohistochemistry showed that the GFP-positive cells were widely distributed in the host brain, including the transplantation site, corpus callosum, neocortex, SVZ, and perilesion area.

The engrafted BMSCs were involved in neurogenesis in the SVZ. Fluorescence immunohistochemistry identified the DCX-positive cells in the SVZ on both sides at 7 weeks after ouabain injection. In the vehicle-treated animals, the number of DCX-positive cells in the SVZ was 34.2 ± 15.0 and 37.2 ± 36.2 in the transplantation and lesion sides, respectively (Fig. 4). BMSC transplantation significantly increased the neurogenesis in the SVZ on both sides. Thus, the number of DCX-positive cells

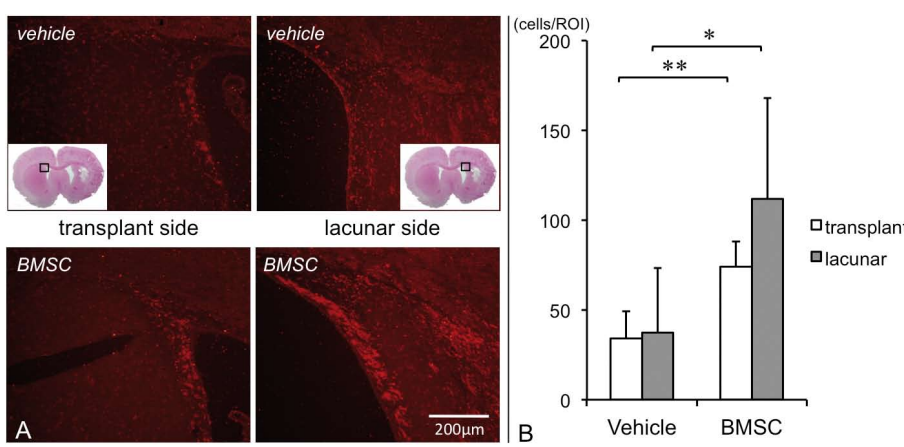

Fig. 4. (A) Photomicrographs of fluorescence immunohistochemistry against doublecortin (DCX) in the subventricular zone (SVZ) in vehicle- (upper) and BMSC-treated animals (lower) at 6 weeks after BMSC transplantation. The right and left panels were photographed in the ouabain-injected and BMSC-transplanted side, respectively. Inset: Photograph demonstrating the area of the brain (box) from which the sample were obtained. (B) Bar graph shows the number of the DCX-positive cells in the SVZ of the vehicle- (left) and BMSC-treated animals (right). The white and gray bars indicate the data in the transplantation side and ouabain-injection side, respectively. *$P<0.05$, **$P<0.01$.

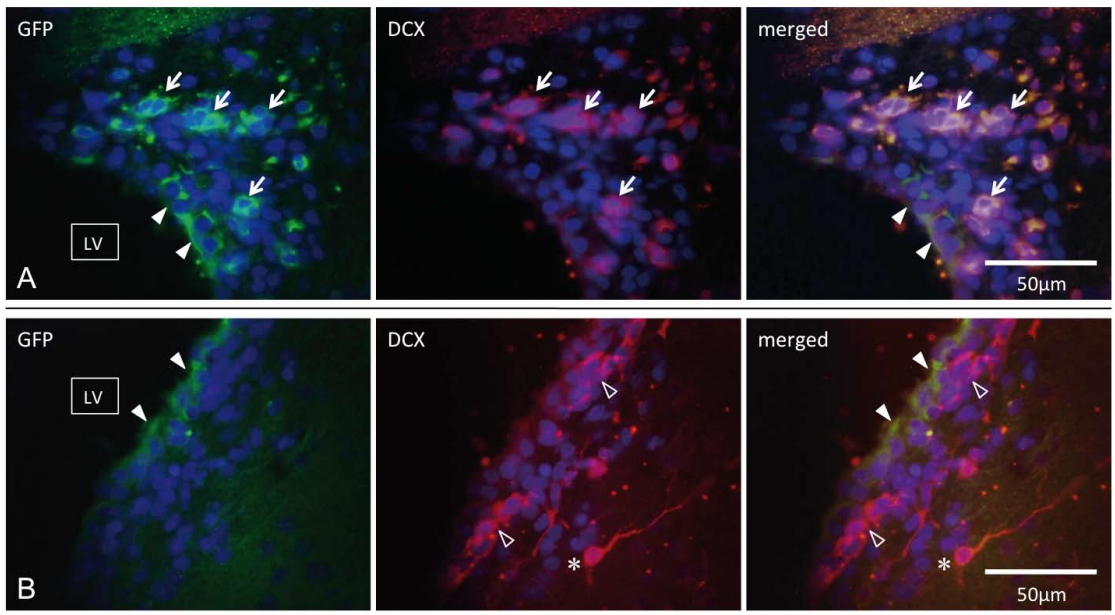

Fig. 5. Photomicrographs of double fluorescence immunohistochemistry against GFP (green) and doublecortin (DCX; red) in the subventricular zone (SVZ) of the BMSC-treated animals at 6 weeks after BMSC transplantation. All photographs were obtained in the SVZ of the BMSC-transplanted side. Arrows, arrowheads, and open arrowheads indicate the $GFP^{+}DCX^{+}$ cells, $GFP^{+}DCX^{-}$ cells and $GFP^{-}DCX^{+}$ cells, respectively. Asterisk in Panel B indicates the DCX-positive cell regarded as a neuroblast. (LV) lateral ventricles.

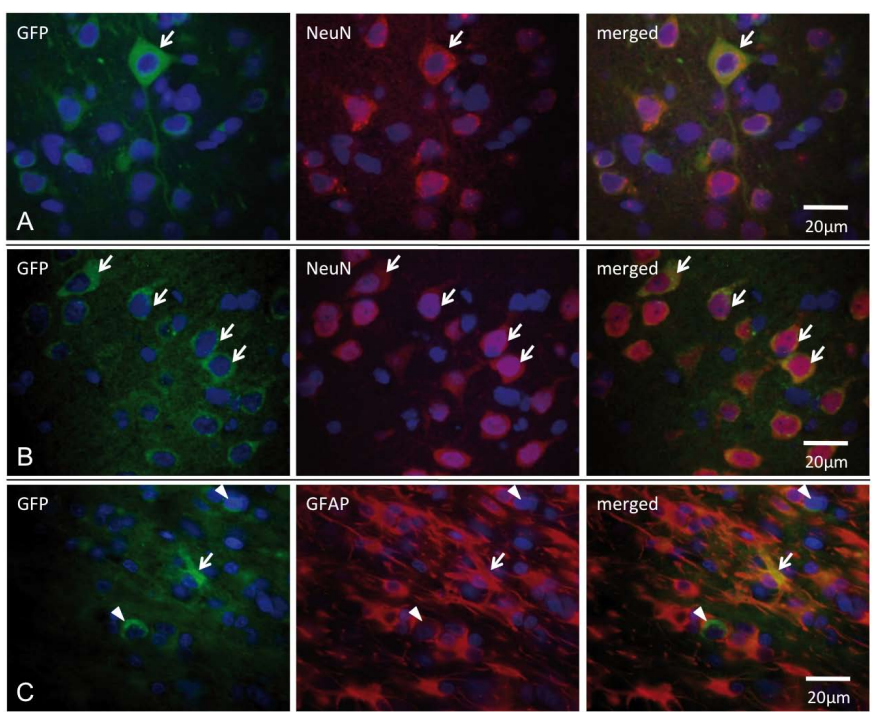

Fig. 6. Photomicrographs of double fluorescence immunohistochemistry against GFP (green), NeuN (A and B, red), and GFAP (C, red) in the BMSC-treated animals at 6 weeks after BMSC transplantation. Panels A, B, C are representative findings in the neocortex of BMSC transplantation side, the neocortex of the ouabain injection side, and the corpus callosum, respectively. Arrows indicate the $GFP^{+}NeuN^{+}$ cells (A and B) and the $GFP^{+}GFAP^{+}$ cells (C). Arrowheads in Panel C indicate the $GFP^{+}GFAP^{-}$ cells.

significantly increased to 74.2 ± 14.0 (P<0.05) and 111.7 ± 36.2 (P<0.01) in the transplantation and lesion sides, respectively (Fig. 4). Double fluorescence immuno- histochemistry showed some of the GFP-expressing BMSCs also expressed DCX, suggesting that the engrafted BMSCs partly contribute to neurogenesis in the SVZ by their neuronal differentiation (Fig. 5).

The BMSCs also migrated towards the neocortex in both hemispheres and some of them expressed NeuN and morphologically simulated the neurons (Fig. 6). The others also expressed GFAP in the corpus callosum (Fig. 6).

DISCUSSION

In this study, the ouabain injection model of rats was employed to verify therapeutic significance of BMSC transplantation on lacunar stroke in humans. Stereotactic injection of ouabain could yield reproducible lesion in the striatum on MRI and histological analysis. The lesion could induce continuous motor deficits lasting for longer than one month. Therefore, the model was considered to simulate lacunar stroke in humans. When the BMSCs were transplanted into the contralateral striatum, motor function significantly improved on rotarod assessment. MR imaging could non-invasively and serially track the SPIO-labeled BMSCs in the host brain. They aggressively migrated towards the ouabain-induced lesion through the corpus callosum within two weeks. Histological analysis clearly showed that the BMSCs were more widely engrafted in the host brain, including the neocortex, corpus callosum, SVZ, and peri-lesion area, than identified on MR imaging. They significantly enhance the neurogenesis in the SVZ and some of them were also involved in neural differentiation in the host brain.

Previously, a variety of animal models have been developed to assess therapeutic effects of drug and cell therapy on ischemic stroke (Stroke Therapy Academic Industry Roundtable 1999, Savitz and Fisher 2007, Savitz et al. 2011). Especially, it is quite important to select the appropriate models relevant to ischemic stroke in human when evaluating the beneficial impacts of cell therapy on ischemic stroke (Savitz et al. 2011). In previous studies, however, the rodent models of middle cerebral artery (MCA) occlusion have been employed in the majority of animal experiments for this purpose. Stereotactic transplantation of the BMSC is known effective to enhance functional recovery after MCA occlusion in rats (Sugiyama et al. 2011b, Kawabori et al. 2012, 2013, Miyamoto et al. 2013). However, there are few animal models that can be employed to denote the effects of cell therapy on lacunar stroke that accounts for about 25% of ischemic stroke (Frost et al. 2006, Janowski et al. 2008). Frost et al. developed a rat model of capsular infarct by injecting endothelin-1 into the brain. They stereotactically injected small amount of endothelin-1, a potent vasoconstrictor peptide, into the internal capsule, and a lacunar infarct-like lesion in the white matter of internal capsule underlying the sensorimotor cortex. However, motor deficits persisted for only two weeks probably because of the small lesion in the white matter (Frost et al. 2006). Subsequently, Janowski and coworkers (2008) have developed alternative rat model of lacunar stroke. They stereotactically injected ouabain, a Na/K-ATPase pump inhibitor, into the striatum. As the results, the lesion involved the basal ganglia, basal forebrain nuclei, internal capsule and striatum (just 1–2% of total brain volume). Significant and relatively stable behavioral deficits were observed for up to 30 days (Janowski et al. 2008). Indeed, the vehicle-treated animals exhibited motor dysfunction for about 50 days in this study. Ouabain is a neurotoxin, but not a vasoconstrictor, and the ouabain-induced lesion is not essentially ischemic. However, ouabain is considered to induce excitotoxicity secondary to cellular membrane depolarization and may closely mimic the pathophysiologic processes of ischemia-induced brain injury (Veldhuis et al. 2003). In this study, therefore, we employed this model to evaluate the effects of BMSC transplantation on lacunar stroke.

This is the first report to show that BMSC transplantation enhances functional recovery in the animal model of lacunar stroke. The underlying mechanisms through which the engrafted BMSCs contribute to functional recovery after various neurological disorders are not fully understood. However, recent studies have strongly suggested that the BMSCs may regenerate the lost neurologic function through multiple biological properties, including neural differentiation, neurotrophic factor release,

and cell fusion around the lesion (Hokari et al. 2008, Kuroda 2013). Furthermore, this study clearly shows that the engrafted BMSCs migrate into the SVZ and enhance intrinsic neurogenesis in the host brain. It is well known that neurogenesis can occur even in the SVZ of adult rodent brain (Alvarez-Buylla et al. 2001, Zhang et al. 2008). Interestingly, neurogenesis is significantly enhanced bilaterally after global and focal experimental ischemia in rodents (Taupin 2006). In this study, the engrafted BMSCs migrate into the SVZ and significantly increase the number of DCX-positive cells. Some of them are also positive for DCX. The findings strongly suggest that the BMSCs differentiate into the DCX-positive cells in the SVZ and simultaneously enhance neurogenesis in the SVZ. Previous studies have suggested that the BMSCs have the potential to differentiate into multilineage cells when exposed to the appropriate environmental cues (Azizi et al. 1998, Kopen et al. 1999, Jendelova et al. 2004). The BMSCs express neuronal phenotype in the neocortex and hippocampus, but express astrocytic phenotype in the corpus callosum when transplanted into the adult rat brain (Lee et al. 2003, Shichinohe et al. 2004). Simultaneously, the BMSCs *per se* are known to support the homing and proliferation of the hematopoietic stem cells in the bone marrow by producing a variety of cytokines such as stromal cell-derived factor (SDF)-1α (Kortesidis et al. 2005). Recent studies have shown that the BMSCs release brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and hepatocyte growth factor (HGF), ameliorating the damage of host neurons (Hokari et al. 2008). Therefore, a certain subpopulation of the BMSCs may support the proliferation and differentiation of neural stem cells in the SVZ through so-called 'nursing effect' (Abe et al. 2012, Kuroda 2013).

In this study, MRI was quite useful to serially track the engrafted cells in the living animals, suggesting the utility in clinical application of cell therapy for ischemic stroke. Indeed, previous study has shown that a 3.0-Tesla clinical MR apparatus can identify minimally one thousand of SPIO-labeled BMSCs as a black spot, using a agar phantom. However, it is still technically difficult to quantify the cell number in the black spot by measuring the intensity on MR imaging probably because of a strong artifact by iron.

CONCLUSIONS

In this study, stereotactic injection of ouabain into the striatum of rat brain induces persistent motor dysfunction for up to 7 weeks and yields the focal lesion mimicking lacunar infarction in the striatum. The BMSCs have the potential to enhance functional recovery after lacunar stroke when stereotactically transplanted into the contralateral hemisphere at clinically relevant timing. Serial MR imaging demonstrate that the engrafted BMSCs aggressively migrate towards the ouabain-induced lesion through the corpus callosum. Histological analysis also shows that the engrafted BMSCs express the neuronal and astrocytic phenotypes in the host brain and also enhance intrinsic neurogenesis in the SVZ.

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