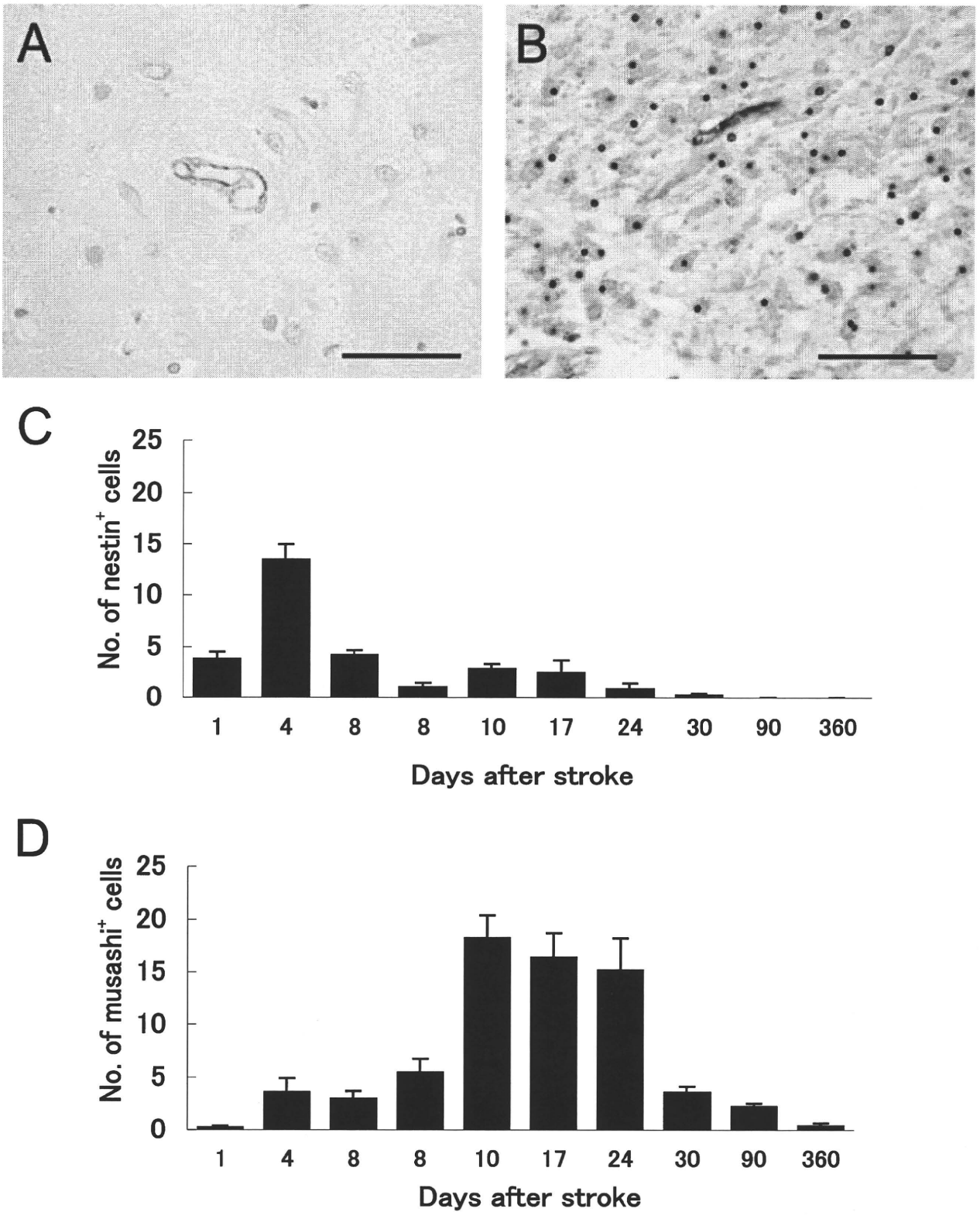

FIG. 4. Temporal profiles of nestin- and musashi-1-positive cells in post-stroke cortex. Panels A and B show nestin-positive cells on day 1 and day 30 post-stroke, respectively. Induction of nestin-positive cells was observed on day 1 in post-stroke cerebral cortex (A), followed by its expression until day 30 (B). Quantitative analysis revealed that the peak number of nestin-positive (C) and musashi-positive cells (D) is at several days and 10–20 days after stoke, respectively. $n = 5$ sections for each time point. Scale bar: 50 μm (A).

anti-CD31 antibody as a marker of endothelial cells. CD31-positive endothelial cells were not observed in the infarcted cortex at this later time point (Fig. 3L).

Temporal profiles of nestin- and musashi-1-positive cells in post-stroke human cortex

Our data thus far indicated that cellular profiles of expression of nestin and musashi-1 varied with regard to location and timing. To investigate these temporal relationships further, nestin- and musashi-1-positive cells were quantified in the area distal from the infarct border (i.e. outside of the area with glial activation) at different time points.

According to this analysis, nestin-positive cells were observed from day 1 after stroke (Fig. 4A) and frequently observed on day 4, followed by decreased expression by day 30 (Fig. 4B). In the later post-stroke period (day 90), no or only very few nestin-positive cells were observed in the previously ischemic area (Fig. 4C). In contrast, although the expression of musashi-1 was observed in some small round cells on day 4 after stroke, its expression appeared to peak from days 10 to 24 post-stroke, subsequently falling off after day 30. Some musashi-positive cells were still observed even 1 year after stroke, although at low levels (Fig. 4D). As a control, brain sections obtained from intact cerebral cortex at each time point were stained with anti-nestin and anti-musashi-1 antibody but no positive cells were observed (not shown).

Discussion

In this study, we have demonstrated induction of potential neural stem/progenitor cells in post-stroke autoptic human cerebral cortex, using autopsied tissue from patients who suffered cardiogenic cerebral embolism. Although the origin of these cells could not be determined, it was unlikely that neural stem/progenitor cells migrated from SVZ or SGZ to the cerebral cortex in such a short period. This supposition is especially relevant to the population of nestin-positive cells observed on day 1 after stroke. Combined with our recent results showing the presence of regional neural stem/progenitor cells in the cerebral cortex following induction of stroke using a rodent model (Nakagomi *et al.*, 2009b), our current observations support the presence of neural stem/progenitor cells in human cerebral cortex that are activated by ischemia.

The presence of neural stem/progenitor cells in SVZ and SGZ is well known, and activation of these stem/progenitor cells by ischemia followed by migration into affected cerebral cortex had been demonstrated in experimental stroke models (Jin *et al.*, 2003; Goings *et al.*, 2004). However, accumulating evidence indicates the presence of neural stem/progenitor cells at various sites in the brain (Arsenijevic *et al.*, 2001; Itoh *et al.*, 2005; Kallur *et al.*, 2006; Willaime-Morawek & van der Kooy, 2008).

In this study, nestin and musashi-1 were used as neural stem/progenitor cell markers. Nestin is a member of the intermediate filament protein family and this marker is abundant in multipotent CNS precursor cells (Lendahl *et al.*, 1990). In adult brain, nestin immunoreactivity is principally observed in SVZ and SGZ (Wei *et al.*, 2002). Increased expression of nestin is seen in a number of pathologic states, including ischemia (Li & Chopp, 1999; Kronenberg *et al.*, 2005; Tonchev *et al.*, 2005), traumatic brain damage (Holmin *et al.*, 1997), inflammation (Cameron & McKay, 2001; Cheeran *et al.*, 2005) and epilepsy (Blumcke *et al.*, 2001). In addition, a recent report has demonstrated the presence of regional neural stem/progenitor cells in post-stroke cerebral cortex that have properties of neural stem/progenitor cells (Nakagomi *et al.*, 2009b). A previous report indicated that nestin-positive cells were observed as early as 6 h and persisted at least 4 weeks after transient focal brain ischemia in a rat stroke model (Li & Chopp, 1999). These observations support the presence of injury-induced neural stem/progenitor cells in human cerebral cortex at diverse sites rather than solely resulting from outmigration of precursors from SVZ and/or SGZ.

Musashi-1-positive cells were also observed in post-stroke cerebral cortex in the current study. Musashi-1 is an RNA binding protein that contributes to maintenance of the stem cell state and differentiation through translational repression of *m-Numb*, the latter an inhibitor of Notch signaling (Sakakibara *et al.*, 1996; Spana & Doe, 1996; Imai *et al.*, 2001). Musashi-1 is mainly expressed in neural progenitor cells, including neural stem cells (Sakakibara *et al.*, 1996; Maslov *et al.*, 2004). Musashi-1-positive neural stem/progenitor cells are induced in SVZ and SGZ after ischemia in experimental stroke models (Yagita *et al.*, 2001; Tonchev *et al.*, 2003, 2005), with a time course that differs from that of nestin-positive cells (Tonchev *et al.*, 2003).

Our study included patients with diverse backgrounds, including age, cardiovascular risk factors and actual infarcted area. Yet we observed a consistent difference in the spatial localization and temporal profile of nestin- versus musashi-1-positive cells in post-stroke cerebral cortex. Although we could not sequentially track differentiation, maturation and migration of nestin- and musashi-1-positive cells in our study of autoptic tissue, our findings are consistent with the heterogeneity of the regenerative response after ischemic injury in humans, a situation analogous to that observed in experimental stroke models (Aguirre *et al.*, 2004; Brazel *et al.*, 2005; Nakagomi *et al.*, 2009b).

GFAP-positive cells are known to include neural stem cells in the SVZ and SZD under physiologic conditions (Kuhn *et al.*, 1996; Alvarez-Buylla & Garcia-Verdugo, 2002). However, abundant injury-induced reactive astrocytes are also well known to express GFAP in the peri-stroke area (Sohur *et al.*, 2006), indicating that GFAP is not, by itself, a sufficient marker to identify injury-induced neurogenesis. In previous reports, nestin has been shown to be expressed not only in neural stem/progenitor cells, but also by immature endothelial cells (Clarke *et al.*, 1994; Gravdal *et al.*, 2009). Similarly, musashi-1 has been detected in endometrial progenitor cells and intestinal stem cells under pathologic conditions (Potten *et al.*, 2003; Gotte *et al.*, 2008). Because there are no absolutely specific markers of neural stem/progenitor cells, it is difficult to be certain that all/most of the nestin- or mushashi-1-positive cells observed in the peri-infarct area are of neuronal lineage (versus representing activation of other cell types in response to the injury imposed by an ischemic event). However, taken together, we believe that our findings provide important insights regarding the temporal profile of immature cell activation (based on evaluation of several cell markers), thereby allowing the design of clinical trials for interventions directed at enhancing the endogenous cell repair mechanisms.

Nestin-positive cells were observed, at least in part, proximal to microvasculature at the periphery of ischemic lesions. Although the precise origin of these cells is unclear, we propose, based on this localization, that injury-induced neural precursors in human cortex may originate, in part, from microvascular pericytes, as has been shown in rodent cortex (Dore-Duffy *et al.*, 2006; Nakagomi *et al.*, 2009b). In addition, this finding may have implications for an important role of the microvasculature in neurogenesis (Ohab *et al.*, 2006; Guo *et al.*, 2008). The influence of microvasculature could reflect the effect of trophic factors and/or formation of a suitable microenvironment, such as stem cell niches. In experimental models,

nestin-positive neural stem/progenitor cells from post-stroke cortex have been shown to form neurosphere-like cell clusters which differentiate *in vitro* into astrocytes, myelin-producing oligodendrocytes and mature neurons with electrophysiologic properties reminiscent of neurons (although no differentiation into mature neurons was observed in the latter study *in vivo*) (Nakagomi *et al.*, 2009b). In the current study, no microvasculature was observed in post-stroke cortex at a later time point (90 days), and the area devoid of microvasculature displayed no mature neurons or glial cells. These findings are consistent with the relatively small contribution that injury-induced neural stem/progenitor cells appear to make to ultimate CNS repair. This is probably because of the lack of the adequate support of neural stem/progenitor cells in the *in vivo* environment of post-stroke cortex. A recent report using a murine model has demonstrated that ischemia induced-neural stem/progenitor cells obtained from post-stroke cortex can be expanded *in vitro* and transplantation of the latter into post-stroke brain enhances functional recover after stroke (Nakagomi *et al.*, 2009a). Taken together, these observations and the results of our current study may provide the basis for a novel cell-based therapy in stroke patients using autologous *ex vivo* expanded stroke-induced neural stem cells. It is important to understand mechanisms of action of potentially therapeutic stem cells as a prelude to clinical trials (Borlongan & Hess, 2006; Borlongan *et al.*, 2008; Borlongan, 2009). Similar to murine stroke models, potential neural stem/progenitor cells were observed in post-stroke human cortex during the acute/sub-acute period in the current study. Our results indicate that stroke treatment targeted to injury-induced neural stem/progenitor cells may be possible beyond the limited 3-h window for fibrinolytic theapy.

In conclusion, our results demonstrate regional activation/induction of neural stem/progenitor cells in affected human cerebral cortex after cardiogenic cerebral embolism during the acute to sub-acute period. In contrast, there is little contribution of the latter cells to CNS repair at longer time points (90 days). These findings indicate the potential value of a therapeutic strategy which appropriately supports injury-induced progenitor cells allowing them to populate, survive and differentiate at the site of a previous ischemic lesion and, thereby, to accelerate the functional recovery of the CNS.

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Abbreviations

CNS, central nervous system; CT, computed tomography; GFAP, glial fibrillary acidic protein; MRI, magnetic resonance image; SVG, subgranular zone; SVZ, subventricular zone.

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Transplantation of cultured choroid plexus epithelial cells via cerebrospinal fluid shows prominent neuroprotective effects against acute ischemic brain injury in the rat

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ABSTRACT

Choroid plexus (CP) epithelial cells (CPECs) produce cerebrospinal fluid (CSF) to provide the CNS with a specialized microenvironment. Our previous study showed that the conditioned medium of cultured CPECs enhanced the survival and neurite extension of hippocampal neurons. The present study examined the ability of cultured CPECs to protect against ischemic brain injury when transplanted into the CSF. Rats were subjected to a transient occlusion of the middle cerebral artery, followed by an injection of cultured CPECs into the fourth ventricle. The injection markedly reduced neurological deficits and infarction volume within 24 h. Other beneficial effects were (1) a reduction in number of apoptotic and inflammatory cells, (2) an up-regulation of the mRNA expression of an anti-apoptotic effecter, cAMP-response element binding protein, and (3) a down-regulation of the production of pro-inflammatory factors such as interleukin-1 beta and inducible nitric oxide synthase. The injected CPECs were located within the ventricles and on the brain's surface, not in the ischemic foci, suggesting that they exert their effects by releasing diffusible neuroprotective factors into the CSF. The transplantation of CPECs via CSF is a potential new strategy for protecting against ischemic brain injury.

Choroid plexus (CP) epithelial cells (CPECs) synthesize a variety of trophic factors including hepatocyte growth factor (HGF) [6], insulin-like growth factor II (IGF-II) [14], and vasculo-endothelial growth factor (VEGF) [4]. Our previous study *in vivo* showed that the grafting of CP into spinal cord lesions promoted tissue repair and nerve regeneration [7], and studies *in vitro* showed that CPECs promoted the extension of neurites in cocultured neurons [3,9]. Subsequently, Watanabe et al. [20] showed that the conditioned medium of cultured CPECs promoted the survival and neurite extension of hippocampal neurons. Matsumoto et al. [12] demonstrated that CPECs produced various functional molecules. There is a close relationship between the CP and brain pathology including Alzheimer's disease [5,18] and ischemic brain injuries [8,9,10,19].

The present study showed that the infusion of CPECs markedly suppressed ischemic damage to the brain, probably through the supply of diffusible factors. This study was previously reported in an abstract form [13].

Male Wistar rats (4–8-week-old) were used. This experiment was approved by the Animal Research Committee, Kyoto University Graduate School of Medicine. All efforts were made to minimize the number of animals and their suffering throughout the experiments.

CPECs were cultured based on the methods of Zheng et al. [22] with some modifications. CP tissues were excised from the lateral and 4th ventricles of 4-week-old rats, dissociated with 0.2% pronase and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 20 μM cytosine arabinoside (Ara-C, to suppress the proliferation of fibroblasts) (Sigma). Five

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days later, the medium was changed to serum-free DMEM without Ara-C. After incubation for an additional 7 days, the cells were used for transplantation. For transplantation, cultured CPECs were labeled by incubation with 1 μg/ml of Hoechst 33342 (Sigma) in DMEM for 30 min, lifted with a 0.4% pronase, and suspended in Hank's balanced salt solution (HBSS) at a density of 1×10^5 cells/μl.

Middle cerebral artery occlusion (MCAO) was performed using the intraluminal filament technique [11] with some modifications. Eight-week-old rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Rectal temperature was maintained at 37 ± 0.5 °C with a rectal thermostat probe. Two holes (1 mm in diameter) were drilled into the skull: one was positioned 1 mm caudal to the bregma and 5 mm lateral to the midline, and the other, 3.8 mm caudal to the lamda suture in the midline. The former hole was for the measurement of regional cerebral blood flow (rCBF) by a laser Doppler Flow Meter (ALF21, Advance, Tokyo, Japan) on the dura of the right parietal cortex. The other hole was used to inject cultured CPECs into the 4th ventricle with an insulin syringe inserted at a depth of 7.0 mm. The right femoral artery was used for recording blood pressure and sampling blood. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical incision. The ECA was transected along with the terminal branches of the lingual and maxillary arteries to make a mobilized stump. A 4-0 nylon monofilament whose distal edge had been coated with paraffin (diameter adjusted to approximately 0.25 mm) was inserted from the ECA stump into the ICA and advanced cranially to the origin of the MCA, until resistance was felt and a slight curving of the filament was observed in the ICA lumen.

Cell transplantation was performed 30 min after MCAO. Cultured CPECs (6×10^6) in 60 μl of HBSS were stereotaxically injected slowly (over 5 min) into the 4th ventricle. The rats were reperfused 30 or 90 min after the injection (i.e., 60 or 120 min after the induction of ischemia) (Fig. 1A).

Physiological parameters including mean arterial blood pressure, arterial blood gas pressures (pO_2 and pCO_2), pH, and rCBF were measured before the operation, 5 min after the induction of ischemia, 5 min after the injection of cells, and 5 min after reperfusion. The "vehicle-injected group" received the same volume of HBSS but without CPECs.

For the behavioral assessment, neurological defects at 24 h after MCAO were evaluated using the neurological severity score (6-point scale) [1]: 0 = no neurological deficit, 1 = failure to extend the left forepaw, 2 = decreased resistance to lateral push, 3 = presence of circling movement, 4 = falling to the left, and 5 = absence of spontaneous motor activity.

For the measurement of infarcted volume, animals were anesthetized and decapitated 24 h after MCAO. Seven serial coronal sections (1.5-mm-thick) of the brain between 3 and 12 mm distal to the frontal pole were stained with a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 20 min, and immersed in a 2% PFA solution. The infarcted (TTC-negative) areas of the cerebral cortex and striatum were quantified using Scion Imaging software, and expressed as the "corrected infarcted area (*A*)" with the equation $A = B - (C - D)$, where *B* represents the area of the cerebral cortex (or striatum) in the left, untreated hemisphere, *C* is the area of the cerebral cortex (or striatum) in the right, treated hemisphere, and *D* is the infarcted area: the infarction volume was calculated by multiplying the infarcted areas by the depth of the infarction and presented as a ratio to the volume of the corresponding contralateral cerebral cortex or striatum.

For the immunohistochemical analysis of cultured CPECs, cells were fixed with 4% paraforaldehyde (PFA) in 0.1 M phosphate buffer (PB), and examined by immunohistochemistry

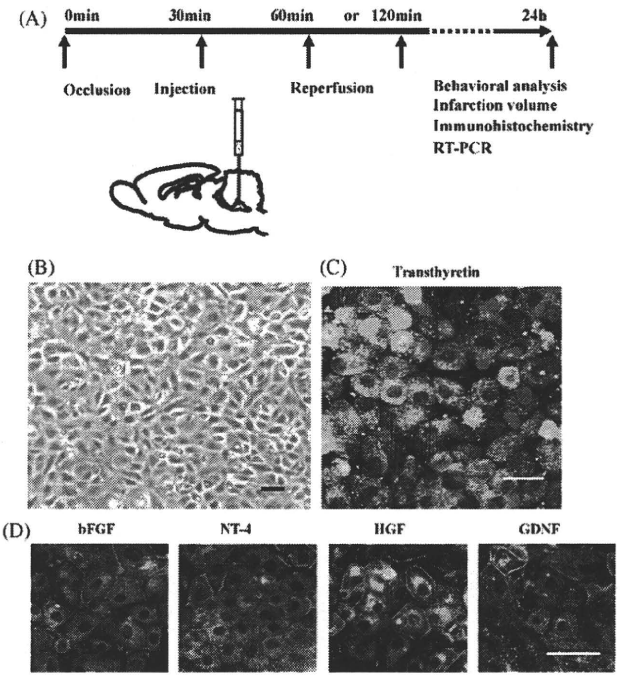

Fig. 1. (A) A diagram outlining the study: occlusion of the middle cerebral artery, injection of CPECs, reperfusion, and analysis of the effects of CPEC-transplantation. (B) Primary cultures of CPECs. (C) Immunoreactivity for transthyretin. Nuclei were stained with TO-PRO-3. (D) Cultured CPECs express bFGF, NT-4, HGF, and GDNF (green). Cell boundaries were highlighted by counterstaining with Phalloidin-TRITC (red). Scale bars: 50 μm.

for transthyretin, thy-1, von Willebrand factor (vWF), basic fibroblast growth factor (bFGF), neurotrophin-4 (NT-4), HGF, and glial cell line-derived neurotrophic factor (GDNF). For immunohistochemistry of brain tissues, rats were perfused transcardially with 4% PFA in 0.1 M PB 24 h after MCAO. Brain tissues were cut into 10-μm-thick coronal sections using a cryostat, and processed for immunohistochemistry for cleaved caspase-3 and OX-42 (a marker of inflammatory cells including macrophages and NK cells). For immunostaining, specimens were incubated for 24 h at 4 °C with primary antibodies against the molecules mentioned above, and, after washing, incubated overnight at 4 °C with secondary antibodies as follows: FITC-conjugated donkey anti-rabbit IgG, FITC-conjugated sheep anti-mouse IgG, and FITC-conjugated rabbit anti-goat IgG (Suppl. 1).

Apoptotic cells were detected in brain sections using the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP–biotin nick-end labeling (TUNEL) assay according to the manufacturer's instructions (Promega, Madison, WI).

Sections were counterstained with TO-PRO-3 (for nuclei, Molecular Probes, 1:1000) and Phalloidin-TRITC (for actin cytoskeleton, Sigma, 1:500), and observed using a confocal scanning laser microscope. The proportion of cells positive for TUNEL or cleaved caspase-3 was measured in the parietal cortex 0.5 mm caudal to the bregma. Data were obtained from 5 animals for each group.

For RT-PCR, tissue blocks of cerebral cortex from the MCA-territory between 3 and 12 mm distal to the frontal pole of the brain were quickly dissected 24 h after MCAO. The total RNA was isolated and quantified spectrophotometrically. One hundred nanograms of total RNA was reverse-transcribed in a 20-μl reaction volume for 30 min at 50 °C. Primer sequences, sizes and amplification cycles are shown in Suppl. 2. PCR products were resolved by electrophoresis, and the relative intensity of the bands on

digitized images of the gel was estimated using Scion Imaging software.

Data were presented as the mean ± SEM. Neurological severity scores were analyzed by nonparametric analysis (Mann–Whitney *U*-test). Physiological parameters (blood pressure, blood gas pressures, pH, and rCBF) during the operation were tested by an analysis of variance (ANOVA) followed by Bonferroni post hoc tests.

More than 95% of the cells were stained with the anti-transthyretin antibody (Fig. 1B and C). None of these cells were immunoreactive with anti-Thy-1 or anti-vWF (data not shown). CPEC cultures did not contain any neurons or other types of glial cells. Immunohistochemistry showed that cultured CPECs expressed trophic factors including basic fibroblast growth factor (bFGF), neurotrophin-4 (NT-4), HGF, and glial cell-derived nerve growth factor (GDNF) (Fig. 1D).

Physiological parameters (blood pressure, blood gas pressure, pH, and rCBF) showed no significant differences between the vehicle-injected and the CPEC-transplanted groups ($n = 7$) (Suppl. 3).

Rats of the CPEC-transplanted group that were reperfused after 60 min of ischemia (60 min MCAO) had smaller infarcted areas in the striatum (43.3 ± 3.4% versus 69.7 ± 2.0%) and cerebral cortex (9.3 ± 3.5% versus 32.9 ± 3.9%), and those that were reperfused after 120 min (120 min MCAO) had smaller infarcted areas in the striatum (58.5 ± 2.7% versus 78.9 ± 2.0%) and cerebral cortex (34.6 ± 7.0% versus 68.9 ± 3.8%) than those of the vehicle-injected group (Fig. 2A–D). The statistics indicated a significant reduction in infarction volume in the CPEC-transplanted group ($n = 10$ for each group in both experiments; $p < 0.01$).

Neurological outcome was markedly improved in the CPEC-transplanted group: the scores were 2.71 ± 0.30 versus 1.21 ± 0.24 ($p < 0.01$; $n = 14$ for each group) in the 60-min MCAO rats (Fig. 2E), and 3.57 ± 0.27 versus 2.43 ± 0.34 ($p < 0.05$; $n = 14$ for each group) in the 120-min MCAO rats (Fig. 2F).

Numbers of apoptotic cells were significantly decreased in the cerebral cortex in the CPEC-transplanted group (43.8 ± 2.5% versus 13.8 ± 1.3%, $n = 5$, $p < 0.01$) (Fig. 3A and B). Similarly, the proportion of cells positive for cleaved caspase-3 in the cerebral cortex was significantly decreased in the CPEC-transplanted group (14.0 ± 1.1% versus 5.4 ± 0.5%, $n = 5$, $p < 0.01$) (Fig. 3C and D).

CPEC-transplantation up-regulated the expression of cAMP-response element binding protein (CREB) and bcl-2, and, in contrast, down-regulated the expression of interleukin-1 beta (IL-1β), iNOS and tumor necrotic factor alpha (TNF-α) (Fig. 4A and B). Whereas extensive infiltration of OX-42-positive cells was found in the affected cerebral areas in the vehicle-injected group,

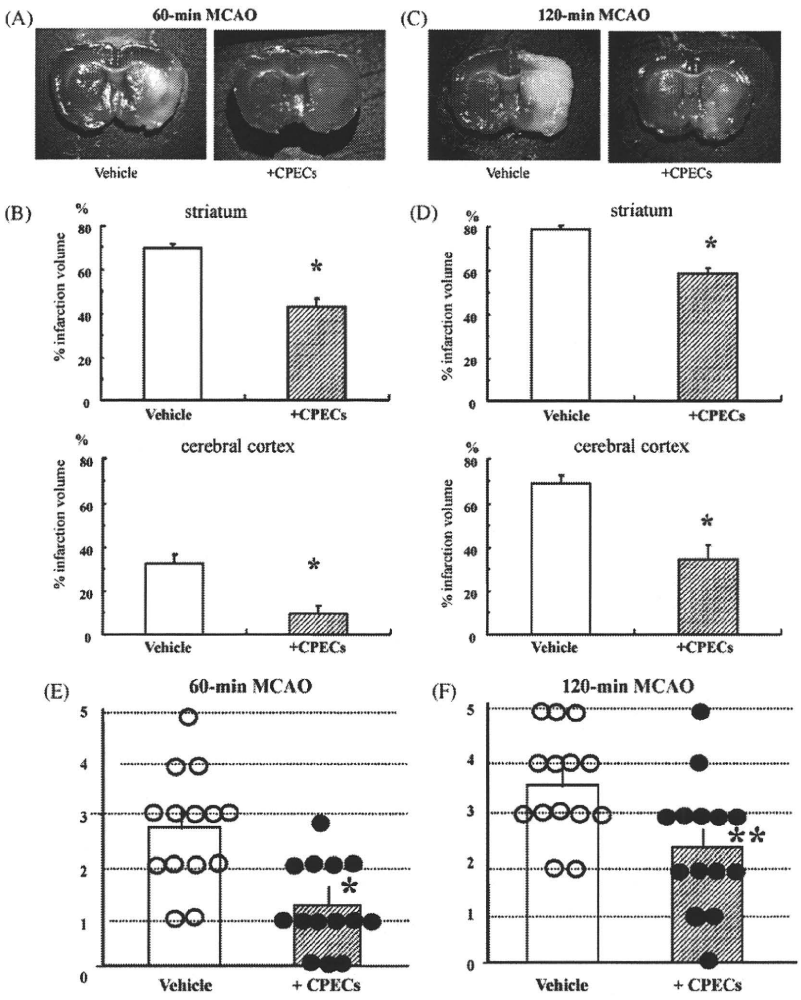

Fig. 2. Infarction volume is markedly reduced in rats with CPEC-transplantation. (A and C) Representative TTC-stained coronal sections from the vehicle-injected group and the CPEC-transplanted group 24 h after a 60-min MCAO (A) and 120-min MCAO (C). (B and D) Infarction volumes are significantly smaller in the CPEC-transplanted rats than vehicle-injected rats, both in the striatum and in the cerebral cortex ($p < 0.01$). (B) and (D) show the result for the 60- and 120-min MCAO, respectively. (E and F) Neurological deficits 24 h after MCAO. Open and closed circles represent the neurological scores of rats from the vehicle-injected and CPEC-transplanted group, respectively. $^{*}p < 0.01$, $^{**}p < 0.05$.

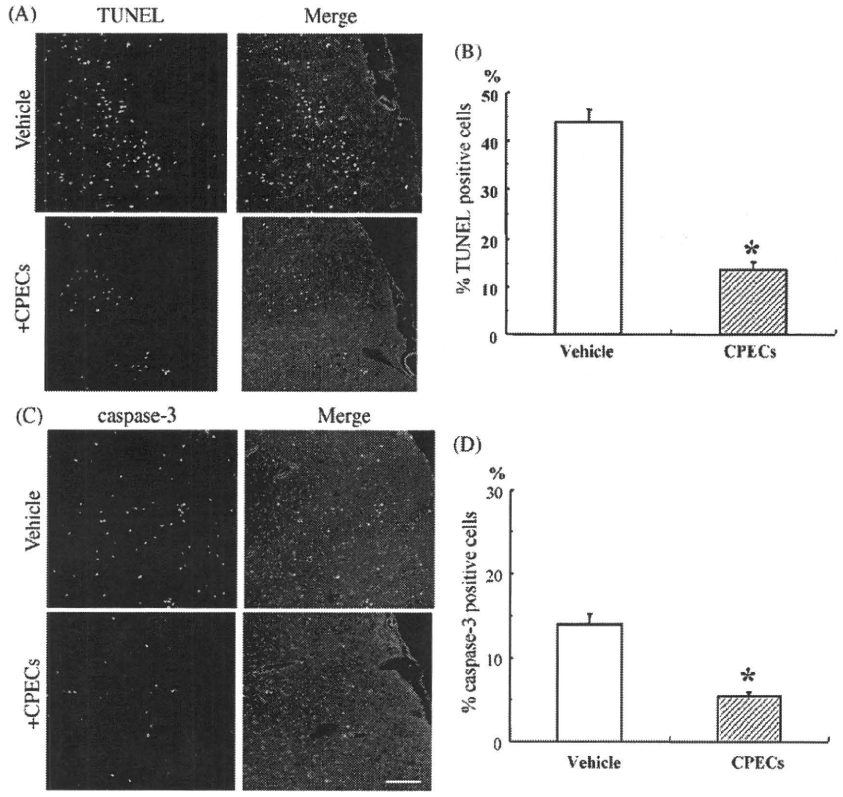

Fig. 3. The transplantation of CPECs suppressed the occurrence of apoptosis after a 120-min MCAO. These pictures are of the affected cerebral cortex 24 h after MCAO. (A and C) There are many TUNEL-positive or caspase-3-positive cells in the cortex in the vehicle-injected group, but far fewer caspase-3-positive cells in the CPEC-transplanted group. Blue: TO-PRO-3, red: Phalloidin-TRITC, green: nuclei of TUNEL-positive cells. (B and D) The occurrence of TUNEL or caspase-3-positive cells was markedly suppressed in the CPEC-transplanted group ($p < 0.01$). Scale bar: 50 μm.

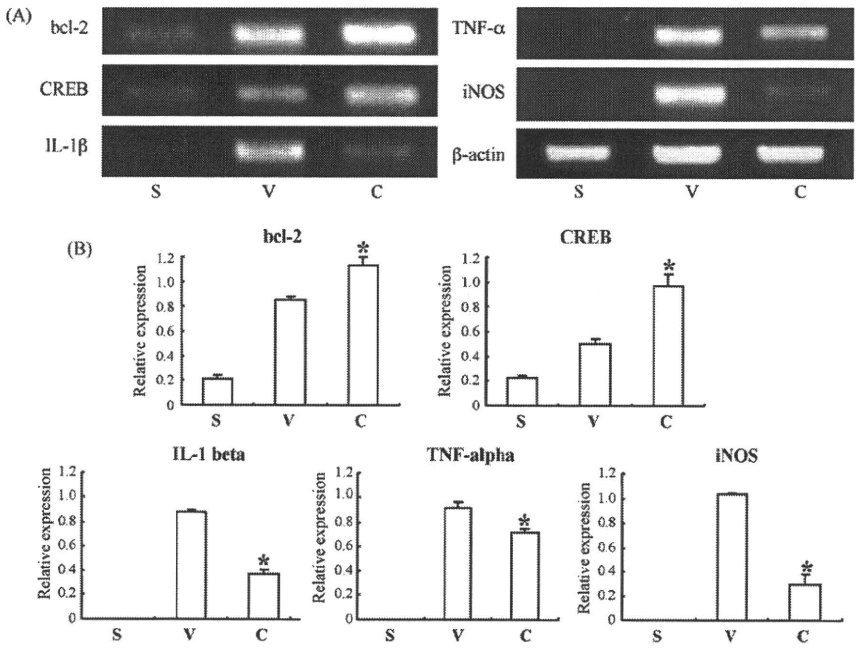

Fig. 4. CPEC-transplantation has profound effects on mRNA levels of bcl-2, CREB, IL-1β, TNF-α and iNOS in the MCAO-injured cerebral cortex. (A) RT-PCR products of bcl-2, CREB, IL-1β, TNF-α, iNOS, and β-actin mRNAs extracted 24 h after MCAO from the affected cerebral cortex of the 120-min MCAO animals: S, sham-operated group; V, vehicle-injected group; C, CPEC-transplanted group. (B) mRNA levels of bcl-2 and CREB were increased and those of IL-1β, TNF-α, and iNOS were decreased in the CPEC-transplanted group compared with the vehicle-injected group. *$p < 0.01$ versus vehicle.

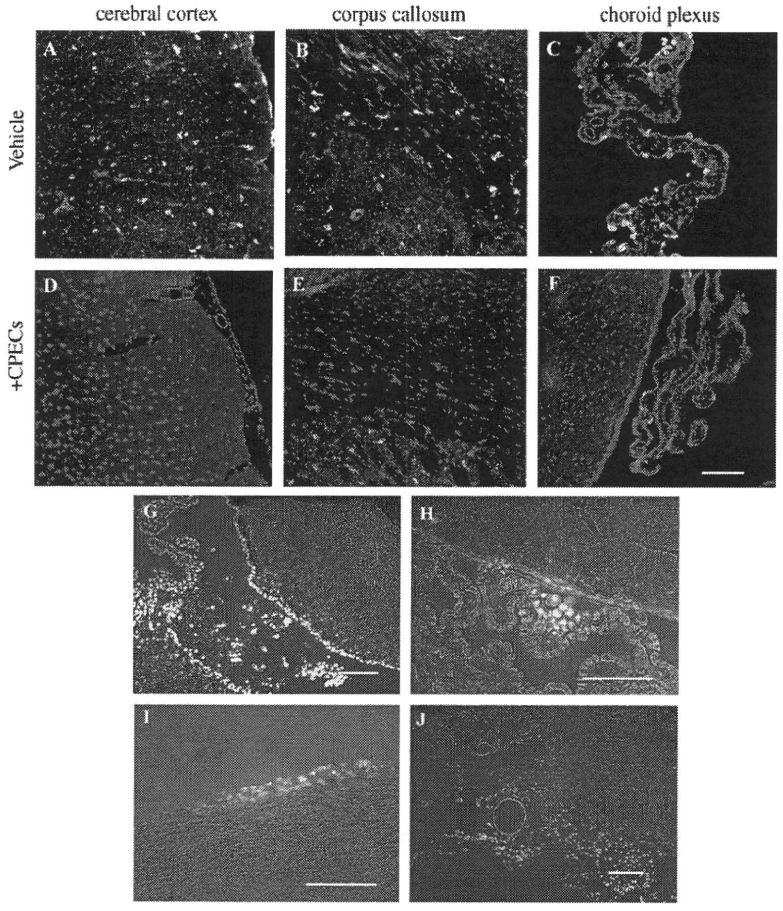

Fig. 5. Inflammatory cells were detected using anti-OX-42 antibody (green). (A and D) cerebral cortex. (B and E) Corpus callosum and a part of the striatum. (C and F) The CP in the lateral ventricle. CPEC-transplantation significantly suppressed the emergence of OX-42-positive cells in the cerebral cortex, corpus callosum, and CP. Blue: TO-PRO-3, and red: Phalloidin-TRITC. Scale bar: 50 μm. (G–J) Hoechst 33342-labeled CPECs (blue) are found on the CP and on ventricular ependymal cells in the 4th (G) and lateral (H) ventricles, at the surface of the medulla (I), and at the base of the hypothalamus (J). Scale bars: 50 μm.

CPEC-transplantation markedly suppressed the appearance of OX-42-positive cells in the cerebral cortex (Fig. 5A and D), corpus callosum (Fig. 5B and E), and CP of the lateral ventricle (Fig. 5C and F).

The Hoechst 33342-labeled CPECs were located in small cell clusters within the lateral and 4th ventricles (Fig. 5G and H) and on the brain's surface (Fig. 5I and J). However, no labeled cells were found at sites of ischemic injury. These transplanted CPECs underwent no apparent morphological changes compared with those in culture.

The present study demonstrated that the transplantation of cultured CPECs into CSF during the acute phase of a transient MCAO dramatically suppressed ischemic brain damage, decreasing infarction volumes, and leading to behavioral improvements 24 h after ischemic injury. Numbers of apoptotic cells and inflammatory cells in the damaged areas were reduced. Anti-apoptotic effectors, CREB and bcl-2, were up-regulated, pro-inflammatory cytokines, IL-1β and TNF-α were down-regulated, and an oxidative stress factor, iNOS, was suppressed at the mRNA level.

None of the transplanted CPECs were found within or in the vicinity of ischemic foci, most of them being found inside the ventricles and on the brain's surface. This strongly suggests that the neuroprotective effect of CPECs was exerted by diffusible factors released from the transplanted cells into the CSF as in the case of bone marrow stromal cell transplantation through CSF [15]. This view is supported by our previous findings *in vitro* [22]. The present study and others show that the CP expresses bFGF, NT-4, GDNF, HGF [6], VEGF [4], and IGF-II [14]. Further analysis of neuroprotective molecules in CSF before and after the infusion of CPECs may be necessary.

CPEC-transplantation suppressed the appearance of TUNEL-positive and cleaved caspase-3-positive cells, and at the same time up-regulated the expression of CREB and bcl-2 in the cerebral cortex. Bone marrow stromal cells also prevent apoptosis and cell death in the ischemic brain. These anti-apoptotic effects of CPECs indicate that CPECs might well be involved in cell survival in the damaged brain tissue. In addition, the suppression of pro-inflammatory molecules such as IL-1β and TNF-α, and appearance of OX-42-positive cells in the cerebral cortex indicate that CPECs have anti-inflammatory effects. Thus, CPEC-transplantation resulted in a concerted up-regulation of the expression of anti-apoptotic genes and down-regulation of pro-inflammatory and oxidative stress-related genes.

Many studies have shown that intravenous application of bone marrow stromal cells has neuroprotective effects on ischemic brain injuries [16]. It is conceivable that CSF-application of cells might be more effective than intravenous application.

A preliminary chronic study indicated that the CPEC-transplantation improved the tissue recovery from ischemic brain damage (Suppl. 4).

Cell injections can be conducted through lumbar puncture [17]. CPECs from the adult CP can be induced to proliferate in culture,

and have the potential to be used as autogenic grafts. Immortalized cell lines of CPECs [21] or encapsulated CP [2] might be another source of transplants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.09.060.

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Bone Marrow Mononuclear Cells Promote Proliferation of Endogenous Neural Stem Cells Through Vascular Niches After Cerebral Infarction

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Key Words. Bone marrow mononuclear cells • Neural stem/progenitor cells • Vascular niches • Endothelial cells • Ischemic stroke

ABSTRACT

Increasing evidence shows that administration of bone marrow mononuclear cells (BMMCs) is a potential treatment for various ischemic diseases, such as ischemic stroke. Although angiogenesis has been considered primarily responsible for the effect of BMMCs, their direct contribution to endothelial cells (ECs) by being a functional elements of vascular niches for neural stem/progenitor cells (NSPCs) has not been considered. Herein, we examine whether BMMCs affected the properties of ECs and NSPCs, and whether they promoted neurogenesis and functional recovery after stroke. We compared i.v. transplantations 1×10^6 BMMCs and phosphate-buffered saline in mice 2 days after cortical infarction. Systemically administered BMMCs preferentially accumulated at the postischemic cortex and peri-infarct area in brains; cell proliferation of ECs (angiogenesis) at these regions was significantly increased in BMMCs-treated mice compared with controls. We also found that endogenous NSPCs developed in close proximity to ECs in and around the poststroke cortex and that ECs were essential for proliferation of these ischemia-induced NSPCs. Furthermore, BMMCs enhanced proliferation of NSPCs as well as ECs. Proliferation of NSPCs was suppressed by additional treatment with endostatin (known to inhibit proliferation of ECs) following BMMCs transplantation. Subsequently, neurogenesis and functional recovery were also promoted in BMMCs-treated mice compared with controls. These results suggest that BMMCs can contribute to the proliferation of endogenous ischemia-induced NSPCs through vascular niche regulation, which includes regulation of endothelial proliferation. In addition, these results suggest that BMMCs transplantation has potential as a novel therapeutic option in stroke treatment. STEM CELLS *2010;28:1292–1302*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neurogenesis is closely associated with stem cell niches in which vascular elements including endothelial cells (ECs) are thought to play a pivotal role. Adult neural stem/progenitor cells (NSPCs) reside in vascular niches in conventional neurogenic zones, such as the subventricular zone (SVZ) of the lateral ventricle [1] and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus [2]. The vasculature is regarded as a key element, especially in the adult SVZ [3], and ECs are thought to contribute importantly to this vascular microenvironment [4, 5]. In support of this viewpoint, coculture experiments have shown that ECs increase proliferation of NSPCs derived from the adult SVZ, thereby promoting neurogenesis [6, 7]. However, accumulating evidence indicates that NSPCs are present in many parts of the adult brain, including the cortex [8–10], subcortical white matter [11], and spinal cord [12–14]; that is, outside conventional neurogenic zones, including SVZ and SGZ. Recently, we also found that NSPCs developed in the poststroke area of the cortex in the adult murine brain (ischemia-induced NSPCs) [8], and that ECs promoted the proliferation of these NSPCs, thereby enhancing neurogenesis after ischemia [15]. These observations suggest that augmentation of ECs (e.g., proliferation of ECs [angiogenesis]) can promote neurogenesis by enhancing the proliferation of endogenous ischemia-induced NSPCs.

Cell transplantation using bone marrow mononuclear cells (BMMCs) has been well-documented to accelerate angiogenesis/neovascularization in the several ischemic diseases such as

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limb ischemia [16, 17] and myocardial infarction [18, 19]. BMMCs contain endothelial progenitor cells (EPCs) [20] that have been shown to contribute to revascularization of ischemic tissues and repair of injured endothelium [21]. Furthermore, BMMCs may have an advantage because they contain several types of bone marrow cells (BMCs), including hematopoietic stem cells (HSCs) [22, 23] and mesenchymal stem cells (MSCs) [24, 25], which can produce large numbers of cytokines and trophic factors that promote central nervous system (CNS) repair after stroke [26, 27]. Accumulating evidence has shown that transplantation of MSCs can reduce infarction size and improve functional outcome in cerebral ischemic animals [28, 29]. However, for clinical use, MSCs require a period of cell culture before transplantation, which increases the risk of contamination and delays the initiation of treatment. In contrast, BMMCs are readily isolated from whole bone marrow by density-gradient centrifugation just before administration and can be used as an autograft. Thus, as an alternative cell source, BMMCs may be a promising form of cell therapy after ischemic stroke. Increasing evidence has shown that BMMCs transplantation reduces infarction size and improves functional outcome in cerebral ischemic animals [25, 30-32]. However, the crucial mechanisms whereby BMMCs exerted CNS repair remain unclear.

In this article, we demonstrate for the first time that ECs are an important element of niches in the cerebral cortex for endogenous ischemia-induced NSPCs and that BMMCs promoted the proliferation of ECs at the ischemic core and the peri-infarct area. We also show that the BMMCs induced effect on ECs accelerated the proliferation of ischemia-induced NSPCs, providing a novel mechanism for BMMCs in neurovascular interaction during cortical repair.

Materials and Methods

Animal Studies

All procedures were carried out under auspices of the Animal Care Committee of Hyogo College of Medicine and National Cardiovascular Center and were in accordance with the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science. Quantitative analyses were conducted by investigators who were blinded to the experimental protocol and identity of samples under study.

Induction of Focal Cerebral Ischemia

Six-week-old male CB-17/Icr-Scid/scid Jcl Mice (SCID mice; Clea Japan Inc., Tokyo, Japan, http://www.clea-japan.com) were subjected to cerebral ischemia. Permanent focal cerebral ischemia was produced by ligation and disconnection of the distal portion of the left middle cerebral artery (MCA) [8, 15, 33, 34]. In brief, the left MCA was isolated, electrocauterized, and disconnected just distal to its crossing of the olfactory tract (distal M1 portion) under halothane inhalation. The infarct area in mice of this background has been shown to be highly reproducible and limited to the ipsilateral cerebral cortex [8, 15, 33, 34].

BMMCs Transplantation in Poststroke Mice

Bone marrow was obtained from 6-week-old normal male C57BL/6 (Japan SLC., Shizuoka, Japan, http://www.jslc.co.jp) or C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131Osb transgenic mice (purchased from RIKEN BRC, Tsukuba, Japan, http://www.brc.riken.go.jp) [35]. Mice were anesthetized with sodium pentobarbital and then sacrificed. The femoral and tibial bones were dissected and bone marrow (BM) was extracted from the bones with serum-free DMEM/F12 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). BMCs were mechanically dissociated into single cells, and BMMCs were isolated by Ficoll-Paque density-gradient centrifugation as described [25, 30, 31]. To suppress rejection of grafted cells, immunodeficient (SCID) mice were used as the recipients of cell transplantation. On poststroke day 2, 1×10^6 BMMCs in 100 μl phosphate-buffered saline (PBS) or control PBS were injected intravenously via the tail vein.

Histological Analysis

Immunohistochemistry was performed as described previously [8, 15]. Detailed conditions are in the Supporting Information Materials and Methods.

Transplantation of ECs into Poststroke Mice

Transplantation of ECs was performed as described previously [15]. Detailed protocol is in the Materials and Methods Section of Supporting Information.

Coculture of ECs with BMMCs Under Direct Cell–Cell Contact Condition

To investigate whether BMMC-induced proliferation of ECs can be observed in vitro, ECs were coincubated under direct cell–cell contact conditions with BMMCs as described previously [15]. A detailed explanation of the conditions is provided in the Materials and Methods Section of Supporting Information.

Culture of ECs with BMMCs-CM

To investigate whether BMMC-derived soluble factors can induce the proliferation of ECs in vitro, ECs were incubated with a BMMCs-conditioned medium (BMMCs-CM) as described previously [26]. A detailed explanation of the conditions is provided in the Materials and Methods Section of Supporting Information.

Measurement of CBF

Cerebral blood flow (CBF) was determined by laser speckle flowmetry (Omegazone laser speckle blood flow imager, Omegawave, Inc, Tokyo, Japan, http://www.omegawave.co.jp/index.html) [36]. A detailed explanation of the conditions is provided in the Materials and Methods Section of Supporting Information.

Statistical Analysis

Results are reported as the mean ± standard deviation (SD). Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA). Where indicated, individual comparisons were performed using Student's t test. Correlations were determined using the Spearman's rank correlation test. Significance was assumed when group differences displayed $p < .05$.

All other methods and materials used in this study are available in the Supporting Information.

Results

Endogenous Ischemia-Induced NSPCs Develop in Close Proximity of ECs

Previously, we found that nestin-positive NSPCs developed in the poststroke cortex of the adult murine brain. Such cells had the capacity for self-renewal and differentiated into

electrophysiologically functional neurons, astrocytes, and myelin-producing oligodendrocytes in vitro [8]. In addition, we have recently shown that ECs promoted the proliferation of these ischemia-induced NSPCs in vitro. Furthermore, we showed that cotransplantation of ECs accelerated the proliferation of grafted exogenous NSPCs [15], indicating that ECs play an important role for the development of ischemia-induced NSPCs. However, the presence of ECs working as vascular niches for the endogenous ischemia-induced NSPCs remains unclear in vivo. First, to examine the colocalization of ECs and NSPCs in and around the poststroke cortex, we performed double immunochemistry for CD31 and nestin on poststroke day 7 (Fig. 1A–1D). Immunohistochemistry showed that most of nestin-positive cells, including those in the ischemic core and the peri-infarct region, were closely associated with CD31-positive ECs (Fig. 1B–1D).

Although nestin is known to be present in neuroepithelial stem cells [37], it has also been shown to be expressed in cells with endothelial phenotype [38]. To confirm that nestin-positive cells developing in close proximity to ECs are NSPCs, microvessels containing perivascular cells (Fig. 1E) from the poststroke area were removed, minced, and incubated in a medium that promotes neurosphere formation [8, 15, 39]. After incubation, large spheres reaching a diameter of approximately 500–800 μm appeared in cultures within 7 days (Fig. 1F). To examine the character of these spheres, they were fixed and cut on a cryostat [8, 15]. Immunohistochemistry using the diaminobenzidine (DAB) reaction showed that CD31 was expressed only in the core of the spheres (Fig. 1G), indicating that the centers of spheres were ECs. In contrast, nestin-positive cells (Fig. 1H) were located at the peripheral zone of the spheres. Double immunohistochemistry showed that nestin-positive cells were present around CD31-positive cells, and coexpression of these markers was not observed (Fig. 1I, 1J). These heterogenous spheres were then dissociated into single cells, and again incubated in the same medium promoting formation of neurospheres [8] at a clonal density of five cells per microliter. On day 20 after incubation, the clonally isolated spheres formed secondary spheres (Fig. 1K). Immunohistochemistry showed that clonal colonies at this density did not contain CD31-positive cells, but rather contained nestin-positive cells (Fig. 1L). Western blot analysis revealed expression of Sox2 (≈34 kDa), a persistent marker for multipotent neural stem cells [40] (Fig. 1M). After differentiation, the cells revealed expression of neuronal (Fig. 1N: MAP2; Fig. 1O: neurofilament; Fig. 1P: Tuj-1), astrocyte [Fig. 1P: glial fibrillary acidic protein (GFAP)] and oligodendrocyte markers [Fig. 1Q: O4; Fig. 1R: myelin-associated glycoprotein (MAG)], which was consistent with the trait of nestin-positive neurospheres (ischemia-induced NSPs) obtained from the poststroke cortex by a conventional method [8, 15]. These observations indicate that some of the ECs, as well as ischemia-induced NSPCs [8], survive in and around the ischemic area even under the ischemic condition and that the NSPCs develop adjacent to ECs.

ECs Induce the Proliferation of Endogenous Ischemia-Induced NSPCs

To further investigate whether ECs are required for proliferation of endogenous ischemia-induced NSPCs developing at the ischemic core and the peri-infarct area, we performed triple immunochemistry for CD31, nestin, and Ki67 on poststroke day 7. Some nestin-positive cells at the ischemic core (≈15%) and the peri-infarct area (≈30%) expressed a dividing cell marker (Ki67) (Fig. 2A), and a large portion (86.8% ± 6.8%) of proliferating ischemia-induced NSPCs (nestin/

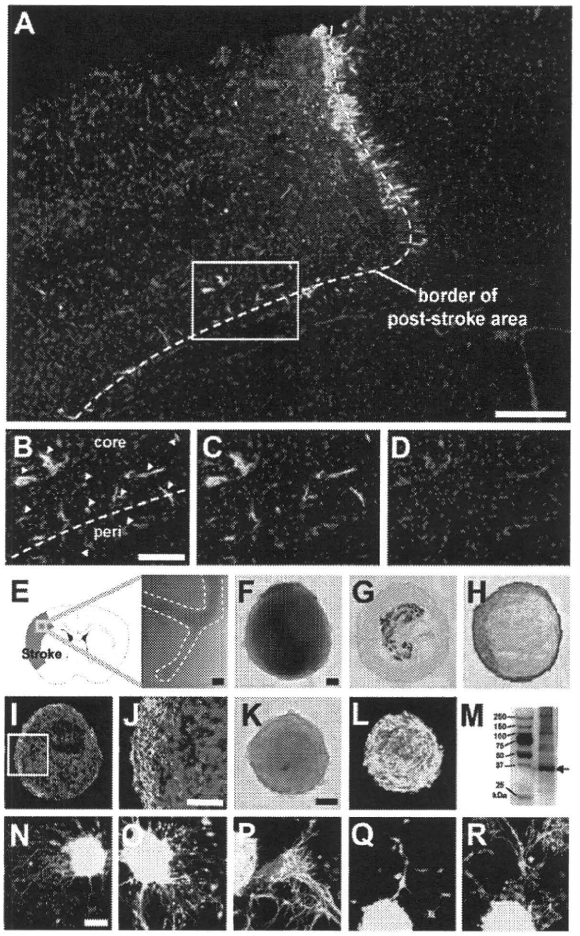

Figure 1. Endogenous ischemia-induced neural stem/progenitor cells (NSPCs) develop in close proximity of endothelial cells (ECs). **(A–D):** Immunohistochemical staining for nestin and CD31 in poststroke mice. On poststroke day 7, immunohistochemistry revealed an expression for nestin and CD31 at the ischemic core and the peri-infarct area [nestin and CD31 (**A,B**); nestin (**C**, green); CD31 (**D**, red); DAPI (**A–D**, blue)]. Note that nestin-positive cells developing at these regions are largely associated with CD31-positive endothelial cells (**B**, arrowheads). **(E–J):** Formation of heterogenous spheres including ECs and NSPCs. Large spheres (**F**) were obtained by cultures of microvascular vessels including perivascular cells in the poststroke cortex (**E**). Immunohistochemistry displayed an expression of CD31 (**G**) in the core and nestin (**H**) in the peripheral zone of the spheres. Double immunohistochemistry showed that CD31 and nestin did not overlap [nestin (**I** and **J**, green); CD31 (**I** and **J**, red); DAPI (**I** and **J**, blue)]. **(K–R):** Formation and characterization of nestin-positive neurospheres. Clonally isolated spheres (**K**) showed nestin but not CD31 [nestin (**L**, green); CD31 (**L**, red); DAPI (**L**, blue)]. Western blot analysis revealed expression of Sox2 in the nestin-positive spheres (**M**, arrow). After differentiation, expression of neuronal (MAP-2 [**N**, green], neurofilament [**O**, green], Tuj-1 [**P**, green], DAPI [**N,O,P**, blue]), astrocyte (GFAP [**P**, red], DAPI [**P**, blue]), and oligodendrocyte markers (O4 [**Q**, green], MAG [**R**, green], DAPI [**Q** and **R**, blue]) was confirmed. Panel **B** shows higher magnification of insets in panel **A** delineated by the white rectangle. Panel **J** shows a higher magnification of the insets in panel **I** delineated by the white square. Scale bar: 500 μm (**A**), 200 μm (**B**), 50 μm (**E**), and 100 μm (**F,J,K,N**). Results displayed are representative of five repetitions of the experimental protocol. Abbreviations: core, ischemic core; DAPI, 4′,6-diamino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; peri, peri-infarct area.

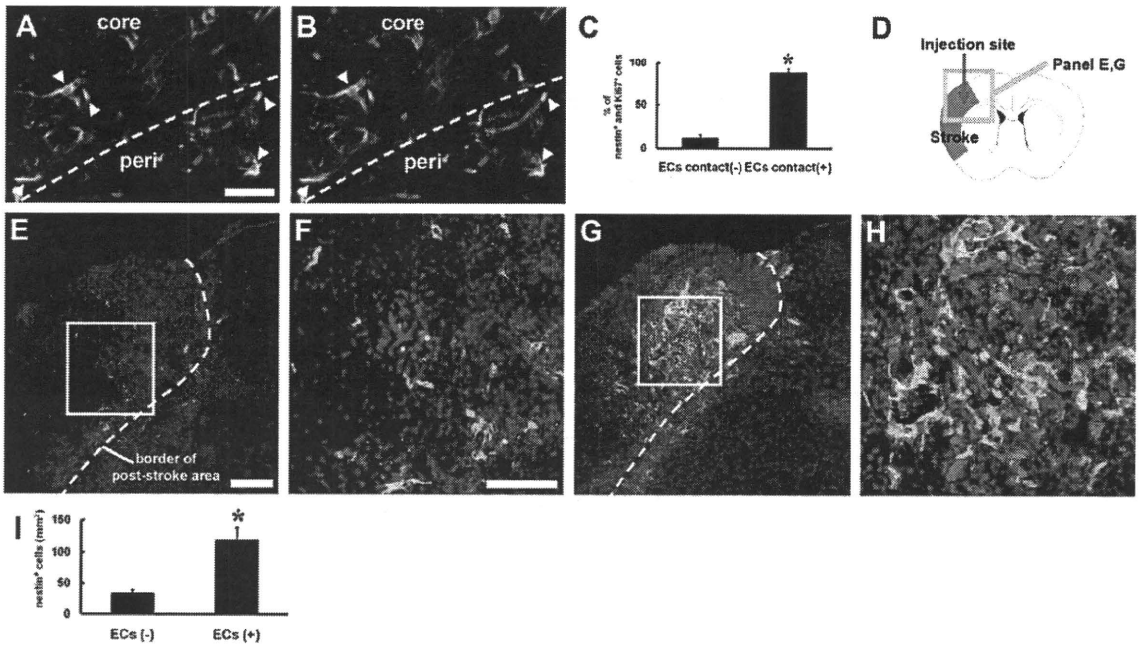

Figure 2. ECs promote the proliferation of endogenous ischemia-induced neural stem/progenitor cells (NSPCs). **(A–C):** Endogenous ischemia-induced NSPCs preferentially proliferated in close proximity to endogenous ECs. On poststroke day 7, immunohistochemistry showed that nestin-positive cells at the ischemic core and the peri-infarct area expressed some Ki67 [nestin (green); Ki67 (blue)](**A**, arrowheads). Note that most of the proliferating neural precursors (nestin/Ki67 double-positive cells) were located in close association with ECs [EC contact (+)] compared with those not in contacting with ECs [nestin (green); CD31 (red); Ki67 (blue) (**B**, arrowheads; **C**). **(D–I):** Transplantation of exogenous ECs accelerated the proliferation of ischemia-induced NSPCs. On poststroke day 7, RFP-positive ECs were transplanted in the poststroke area (**D**). Although a few nestin-positive cells were observed in mice injected only with PBS [nestin (green); DAPI (blue)](**E,F**), many nestin-positive cells were observed near RFP-positive ECs [nestin (green); RFP (red); DAPI (blue)](**G,H**). A significantly increased number of nestin-positive cells were observed in mice with ECs [EC (+)] compared with mice without ECs [EC (−)](**I**). Panels **F** and **H** show higher magnification of insets in panel **E** and **G** indicated by white squares, respectively. $n = 5$ for each experimental group. $^*p < .05$ versus [EC contact (−)](**C**) or [EC (−)](**I**). Scale bar: 100 μm (**A**), 200 μm (**E**), and 100 μm (**F**). Results displayed are representative of five repetitions of the experimental protocol. Abbreviations: core, ischemic core; DAPI, 4′,6-diamino-2-phenylindole; ECs, endothelial cells; peri, peri-infarct area; RFP, red fluorescent protein.

Ki67 double-positive cells) at these regions existed in the proximity of ECs (Fig. 2B, 2C). These observations suggest that vascular niches play an essential role for proliferation of endogenous ischemia-induced NSPCs.

To confirm these findings, we grafted red fluorescent protein (RFP)-positive ECs onto the poststroke cortex of mice (Fig. 2D). Although nestin-positive ischemia-induced NSPCs developed at the ischemic core and the peri-infarct region even in mice without ECs (Fig. 2E, 2F) as described previously [8], mice with ECs displayed a greatly increased number of nestin-positive cells (Fig. 2G, 2H). Note that these nestin-positive cells were closely associated with grafted ECs. Semiquantitative analysis confirmed that the number of nestin-positive cells was significantly increased in mice with ECs compared with control mice (Fig. 2I). Combined with our previous result that ECs could accelerate the proliferation of ischemia-induced NSPCs under coculture conditions [15], these observations indicate that ECs enhance the development and proliferation of endogenous ischemia-induced NSPCs.

Characterization of BMMCs by Flow Cytometry

Next, to investigate the effect of BMMCs on vascular niches, we administered BMMCs into mice subjected to ischemic stroke. First, we investigated the character of mouse BMMCs by flow cytometric analysis. As a result, ≈91% of BMMCs were positive for CD45, a marker of hematopoietic cells (Supporting Information Fig. 1A). Expression of CD3 (T-cell marker) (Supporting Information Fig. 1B), CD45R (B-cell marker) (Supporting Information Fig. 1C), and CD11b (myeloid cell marker) (Supporting Information Fig. 1D) was observed in ≈6%, ≈32%, and ≈27% of BMMCs, respectively. In addition, ≈17% and ≈7% expressed lineage marker-negative (Lin^-) (Supporting Information Fig. 1E) and Sca-1 (Supporting Information Fig. 1F), respectively, in which HSCs are included [41]; ≈22% expressed stem cell marker c-kit (Supporting Information Fig. 1G), which is present on HSCs [42]; ≈11% expressed CD34 (Supporting Information Fig. 1H), which is observed in HSCs and EPCs. These surface marker patterns were consistent with previous analyses on cell populations of BMMCs [22, 23].

BMMCs Accumulate in and Around the Poststroke Cortex

We then analyzed the localization of green fluorescent protein (GFP)-positive BMMCs in brains (Fig. 3A–3D). Immunohistochemistry on poststroke day 3 showed that a substantial amount of GFP-positive BMMCs was detectable in the ischemic core (6.3 ± 1.9 per square millimeter) and the peri-infarct area (15.3 ± 2.9 per square millimeter) (Fig. 3B). Some GFP-positive cells were in contact with ECs at the ischemic core and the peri-stroke area. In contrast, only a few GFP-positive BMMCs were observed in the nonischemic ipsilateral (0.09 ± 0.02 per square millimeter) (Fig. 3C) and contralateral cortices (0.06 ± 0.01 per square millimeter) (Fig. 3D). Semiquantitative analysis showed that most of BMMCs accumulated in and around the ischemic cortex (Fig. 3E). The number of BMMCs preferentially

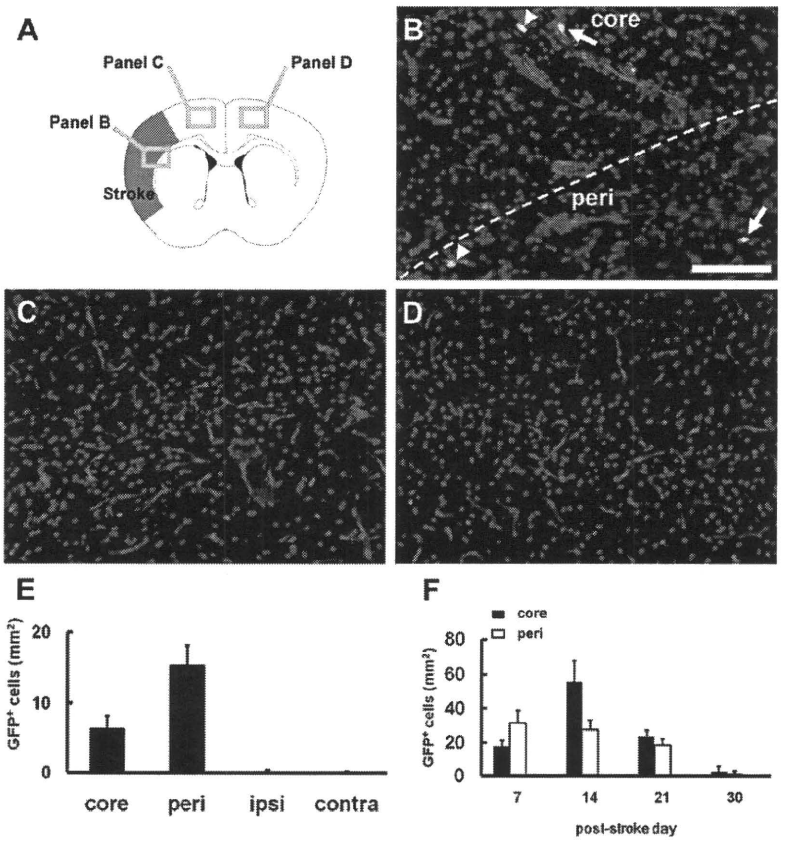

Figure 3. Administered bone marrow mononuclear cells (BMMCs) predominantly accumulate at the ischemic core and the peri-infarct area. **(A–E):** Immunohistochemical analysis of localization of intravenously transplanted BMMCs in brains. One day after BMMCs transplantation, localization of GFP-positive BMMCs was analyzed ($n = 5$) (**A**). Immunohistochemistry showed that GFP-positive BMMCs largely accumulated in and around the poststroke area [GFP (green); CD31 (red); DAPI (blue)](**B**, arrows and arrowheads; **E**), whereas they were rarely observed in the ipsilateral [CD31 (red); DAPI (blue)](**C,E**) and contralateral cortices [CD31 (red); DAPI (blue)](**D,E**). Note that some GFP-positive BMMCs were in contact with ECs at the ischemic core and the peristroke area (**B**, arrowheads). **(F):** Semiquantitative analysis of the number of GFP-positive BMMCs at the ischemic core and peristroke area following transplantation. Scale bar: 200 μm (**B**). Results displayed represent five repetitions of the experimental protocol. Abbreviations: contra, contralateral cortex; core, ischemic core; DAPI, 4′,6-diamino-2-phenylindole; GFP, green fluorescent protein; ipsi, ipsilateral cortex; peri, peri-infarct area.

homed to ischemic regions increased up to 14 days after stroke. Their number then gradually decreased at later time points. (Fig. 3F). Although we observed the transplanted GFP-positive BMMCs until 30 days after stroke, only a few GFP-positive cells (< 1%) exhibited endothelial phenotype (not shown).

BMMCs Increase ECs in and Around the Poststroke Cortex

To investigate whether BMMCs could affect the properties of ECs in and around the poststroke cortex, mice were then subjected to immunohistochemical analysis on poststroke day 7. Because bioactive ECs express endothelial nitric oxide synthase (eNOS), we first examined the expression of eNOS (Supporting Information Fig. 2A–2F). Compared with PBS-injected mice (Supporting Information Fig. 2A–2C), the stronger expression of eNOS was observed at CD31-positive cells of BMMCs-treated mice (Supporting Information Fig. 2D–2F).

We further investigated whether the BMMCs-derived positive effect was attributed to the promotion of cell proliferation or survival for ECs. To examine whether BMMCs can induce the proliferation of ECs (angiogenesis) after stroke, we observed the number of CD31/BrdU double-positive cells at the ischemic core and the peri-stroke region 7 days after MCA occlusion (Fig. 4A, 4B). Although the CD31/BrdU double-positive cells were observed in mice treated with PBS (Fig. 4A) and BMMCs (Fig. 4B), the number of CD31/BrdU double-positive cells had significantly increased at the ischemic core and the peri-stroke region in BMMCs-treated mice compared with PBS-injected mice (Fig. 4C). In addition to these findings, the ratio of CD31/BrdU double-positive cells to CD31-positive cells was significantly increased at the ischemic core (3.2 ± 1.4 and 9.1% ± 1.1% in the PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p < .05$) and the peri-stroke region (11.9 ± 2.6 and 21.6% ± 3.4% in the PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p < .05$) in BMMCs-treated mice compared with those observed in PBS-treated mice.

Furthermore, to investigate whether BMMCs affect the survival of ECs, we observed the number of CD31/caspase-3 double-positive cells 7 days after ischemia (Fig. 4D, 4E). No significant difference was observed between PBS- (Fig. 4D, 4F) and BMMCs-treated mice (Fig. 4E, 4F). Thus, we analyzed the ratio of CD31/caspase3 double-positive cells to CD31-positive cells between the two groups. However, no significant differences were observed at the ischemic core (21.6 ± 4.8 and 17.9% ± 3.7% in the PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p = .20$) and the peri-infarct region (20.7 ± 4.7 and 16.7% ± 3.4% in the PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p = .17$).

To investigate ECs of the ischemic core and the peri-infarct area at a later time, we examined the expression of CD31-positive cells on day 30 poststroke (Fig. 4G, 4H). Although CD31-positive cells were observed, especially at the peri-infarct area in PBS- (Fig. 4G) and BMMCs-treated mice (Fig. 4H), CD31 expression was enhanced in the BMMCs-treated mice (Fig. 4H) compared with the controls (Fig. 4G). In addition to these findings, CD31 and eNOS expression shown by Western blot analysis was increased in BMMCs-treated mice compared with controls (Fig. 4I). These data indicate that BMMCs promoted the proliferation of ECs rather than the survival of ECs in and around the poststroke cortex, thereby augmenting ECs in vivo.

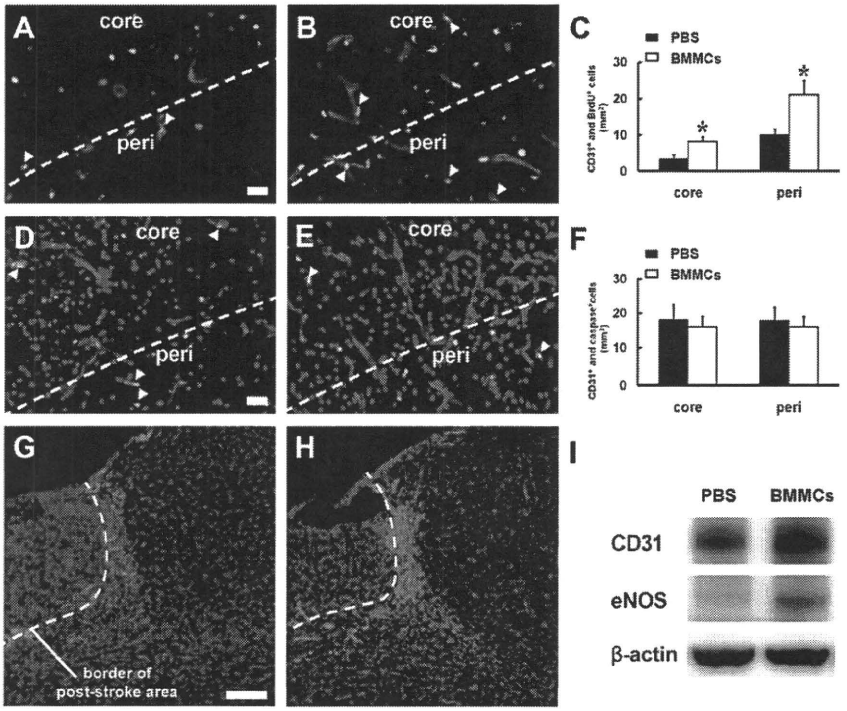

Figure 4. BMMCs transplantation increases endothelial cells (ECs) in and around the stroke-affected cortex. **(A–C):** Immunohistochemical analysis for CD31 and BrdU on poststroke day 7. Compared with PBS-injected mice [CD31 (red); BrdU (green)](**A**, arrowheads), BMMCs promoted the proliferation of ECs [CD31 (red); BrdU (green)](**B**, arrowheads). Semiquantitative analysis displayed that CD31/BrdU double-positive cells were increased at the ischemic core and the peristroke area in BMMCs-treated mice compared with those of controls (**C**). **(D–F):** Immunohistochemical analysis for CD31 and caspase3 on poststroke day 7. Compared with PBS-injected mice [CD31 (red); caspase3 (green); DAPI (blue)](**D**, arrowheads; **F**), there are no significant differences in the number of CD31/caspase3 double-positive cells in the ischemic core and the peri-ischemic region in BMMCs-treated mice [CD31 (red); caspase3 (green); DAPI (blue)](**E**, arrowheads; **F**). **(G–I):** Immunohistochemical analysis of ECs at poststroke day 30. Immunohistochemistry showed that fewer CD31-positive cells were observed in control mice [CD31 (red); DAPI (blue)](**G**), whereas many CD31-positive cells were observed at the peri-ischemic region in BMMCs-treated mice [CD31 (red); DAPI (blue)](**H**). Western blot analysis at the same time also showed that expression of CD31 and eNOS was increased in BMMCs-treated mice compared with controls (**I**). $n = 5$ for each experimental group. $*p < .05$ versus PBS group of each region (**C**). Scale bar: 50 mm (**A,D**) and 200 μm (**G**). Results displayed represent five repetitions of the experimental protocol. Abbreviations: BMMCs, bone marrow mononuclear cells; BrdU, bromodeoxyuridine; core, ischemic core; DAPI, 4′,6-diamino-2-phenylindole; eNOS, endothelial nitric oxide synthase; PBS, phosphate buffered saline; peri, peri-infarct area.

BMMCs Increase CBF in the Poststroke Mice

To further examine whether BMMCs could increase microvascular circulation, we analyzed regional CBF. Immediately after injection of BMMCs or PBS (poststroke day 2), there was no significant difference in CBF between the two groups. However, 30 days after the MCA occlusion, CBF was significantly increased in BMMCs-treated mice compared with that in controls (Fig. 5A, 5B). These observations suggest that endothelial proliferation may partly result in functional changes in cerebral circulation.

BMMCs Promote the Proliferation of ECs In Vitro

To investigate whether BMMCs-induced proliferation of ECs can also be observed in vitro, we cocultured the RFP-positive ECs with GFP-positive BMMCs under direct cell–cell contact conditions. Compared with ECs without BMMCs (Fig. 6A), ECs cocultured with BMMCs displayed an increase in cell density and BrdU incorporation (Fig. 6B). This impression was confirmed by quantitative analysis of the number of RFP-positive cells (Fig. 6C) or ratio of RFP/BrdU double-positive cells to RFP-positive cells (Fig. 6D). The effect of proliferation of ECs by BMMCs appears to be dose-dependent (Fig. 6E).

To further investigate whether BMMCs affected cell survival of ECs in vitro, we examined the expression of caspase3 on ECs cocultured with or without BMMCs. As a result, there was no significant difference in the ratio of RFP/caspase3 double-positive cells to RFP-positive cells between EC alone (Fig. 6F) and ECs with BMMCs (Fig. 6G) under the current culture conditions (Fig. 6H). These observations indicate that an increase of ECs is not likely caused by a suppression of cell death, but rather, is a result of enhanced proliferation of ECs.

Thus, we cocultured ECs with BMMCs under direct cell–cell contact conditions and found that cocultured ECs increased 4.4-fold compared with those cultured alone (Fig. 6A–6C). Under conditions in which the contact between BMMCs and ECs occurs, the proliferation of ECs is dependent on direct cell–cell contact regulation and BMMCs-derived soluble factors. We incubated ECs with BMMCs-CM and found that the number of RFP-positive ECs increased 3.4-fold compared with that of ECs in the control medium (CM) (Fig. 6I). However, the number of ECs cultured alone (48.2 ± 5) and those treated with CM (42.6 ± 5.5) was not significantly different. The number of RFP-positive ECs increased significantly under direct cell–cell contact conditions with BMMCs (212.4 ± 22.2) compared with those stimulated with BMMCs-CM (145.2 ± 10.6) ($p < .05$). Although the maximum impact of BMMCs on ECs was observed under direct cell–cell contact conditions, BMMCs-CM alone could increase the number of ECs to approximately 70% compared

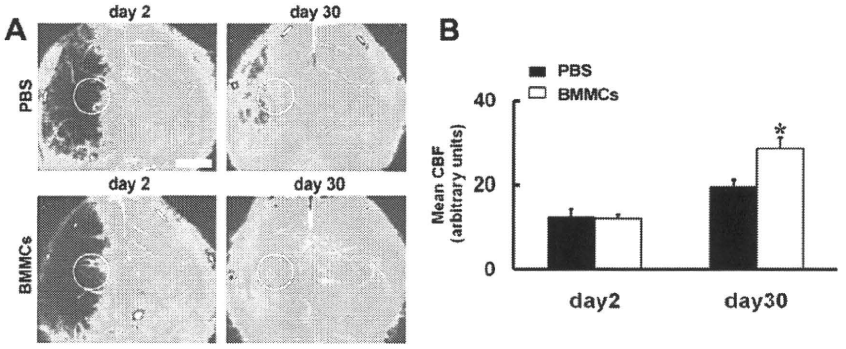

Figure 5. CBF analysis in poststroke mice. **(A,B):** Mean CBF within region of interest (white circled area) was measured at poststroke day 2 or 30 in mice treated with PBS or BMMCs (**A**). No significant difference was observed between two groups immediately after treatment. However, 30 days after middle cerebral artery occlusion, CBF was significantly increased in BMMCs-treated mice compared with controls (**B**). $n = 5$ for each experimental group. $*p < .05$ versus PBS group at the same time point (**B**). Scale bar: 2 mm (**A**). Results displayed are representative of five repetitions of the experimental protocol. Abbreviations: BMMCs, bone marrow mononuclear cells; CBF, cerebral blood flow; PBS, phosphate buffered saline.

with the direct cell–cell contact conditions. Similar to these findings, BMMCs-CM induced the proliferation of several types of ECs, such as human pulmonary microvascular ECs (hp-ECs) (3.2-fold) and mouse brain ECs (mb-ECs) (3.1-fold) compared with the controls (Supporting Information Fig. 3). These observations indicate that BMMCs-induced proliferation of ECs is partly caused by soluble factors. Thus, we further investigated the concentration of a variety of trophic factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and fibroblast growth factor-basic (FGF-2), with angiogenic and mitogenic effects. Compared with CM, the value of EGF or FGF-2 was not significantly changed in the BMMCs-CM (Fig. 6J). However, VEGF or IGF-1 levels were significantly higher in the BMMCs-CM compared with CM (Fig. 6J). To examine whether these growth factors can affect the proliferation of ECs, we incubated ECs with BMMCs-CM that was pretreated with anti-VEGF- (Fig. 6K) or IGF-1-neutralizing antibody (Fig. 6L), and found that number of ECs was lowered by treatment with these antibodies (Fig. 6K, 6L). These findings showed that BMMCs-derived trophic factors contribute, at least to some extent, to the proliferation of ECs.

BMMCs Promotes the Proliferation of Ischemia-Induced NSPs and Accelerates Neurogenesis with Functional Recovery

Thus far, our data had indicated that ECs were essential for proliferation of endogenous ischemia-induced NSPCs and also showed that BMMCs promoted the proliferation of ECs, thereby increasing ECs in vivo and in vitro.

To study whether BMMCs-induced augmentation of ECs can promote the proliferation of ischemia-induced NSPCs in vivo, we compared the number of nestin/BrdU double-positive cells at the ischemic core and the peri-infarct area after treatment with PBS and BMMCs (Fig. 7A, 7B). Immunohistochemistry 7 days after MCA occlusion showed that, compared with PBS-injected mice (Fig. 7A, 7D), a larger number of nestin/BrdU double-positive cells were observed at the ischemic core and the peri-infarct region of BMMCs-treated mice (Fig. 7B, 7D). Similar to these results, proliferation of the NSPCs assessed by the ratio of nestin/BrdU double-positive cells to nestin-positive cells in BMMCs-treated mice was significantly increased at the ischemic core (12.8 ± 4.1 and 20.2% ± 2.6% in PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p < .05$) and the peri-infarct region (28.9 ± 3.8 and 42.9% ± 2.8% in PBS-injected and BMMCs-treated groups, respectively; $n = 5$ per group, $p < .05$) compared with that observed in controls. Moreover, to confirm the hypothesis that proliferation of ischemia-induced NSPCs was attributable to a BMMCs-induced increase of ECs by endothelial proliferation (angiogenesis), we transplanted BMMCs onto postischemic mice and then administered endostatin (an antiangiogenic agent known to inhibit proliferation of ECs [43]) daily. Compared with mice treated with only BMMCs, the number of nestin/BrdU double-positive cells was significantly decreased by mice receiving additional treatment with endostatin following BMMCs transplantation (BMMCs + endostatin) (Fig. 7C, 7D). In mice treated with BMMCs plus endostatin, it was confirmed that proliferation of ECs assessed by the number of CD31/BrdU double-positive cells had significantly decreased at the ischemic core (8.4 ± 1.3 and 4.4 ± 1.1 cells per square millimeter in the BMMCs and BMMCs + endostatin groups, respectively; $n = 5$ per group, $p < .05$) and the peri-infarct region (20.2 ± 3.1 and 13.4 ± 2.0 cells per square millimeter in the BMMCs and BMMCs + endostatin groups, respectively; $n = 5$ per group, $p < .05$) compared with that observed in BMMCs-treated mice. These findings indicate that BMMCs promote the proliferation of endogenous ischemia-induced NSPCs through vascular niche regulation, at least in part by an increase in ECs resulting from endothelial proliferation (angiogenesis).

Finally, we assessed whether BMMCs could promote neurogenesis and relieve functional impairment after stroke. Previously, we had demonstrated that ischemia-induced NSPCs that developed in the poststroke cortex migrated toward the peri-infarct area [8], and that they could partially differentiate into neurons at that area, especially under the influence of ECs [15]. Based on these observations, we assessed the number of NeuN/BrdU double-positive cells at the peri-infarct region on poststroke day 30 (Fig. 7E–7H). As a result, a lower number of NeuN/BrdU double-positive cells was observed in control mice (Fig. 7E, 7H), whereas a larger number of NeuN/BrdU double-positive cells was observed in BMMCs-treated mice (Fig. 7F, 7H). However, compared with BMMCs-treated mice, NeuN/BrdU double-positive cells were significantly suppressed in mice treated with BMMCs plus endostatin (Fig. 7G, 7H).

Functional recovery of mice subjected to stroke and then treated with BMMCs was investigated by behavioral testing using a modification of the open field task [44] as described [15, 33, 34]. Because dysfunction of the cortex is closely linked to disinhibition of behavior in the presence of light [15, 33], we investigated locomotion during the light phase 30 days after stroke. Compared with PBS-injected mice,

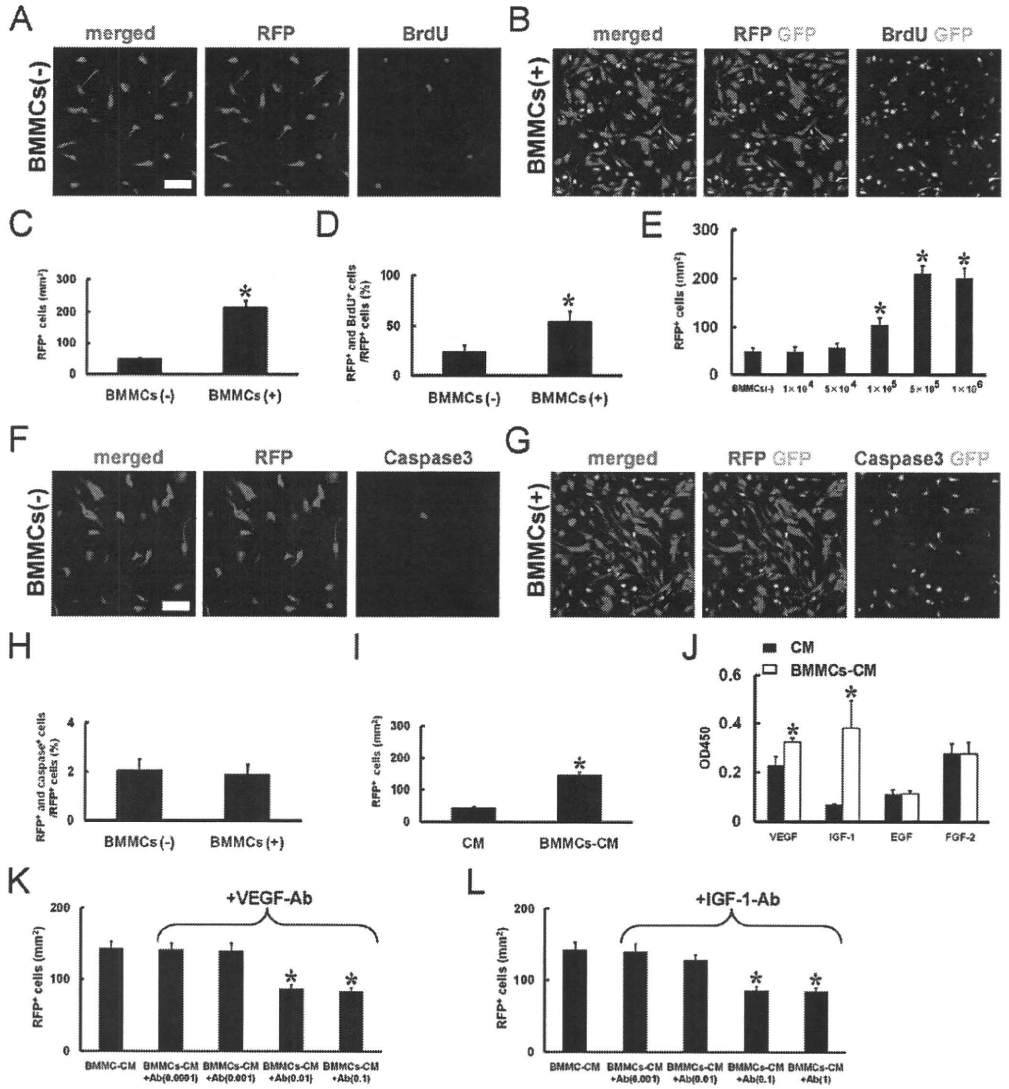

Figure 6. BMMCs accelerate the proliferation of endothelial cells (ECs) in vitro. **(A–E):** BMMCs promoted the proliferation of ECs in vitro. Compared with RFP-positive ECs without BMMCs [BMMCs (−)][RFP (red); BrdU (blue)](**A,C,D**), RFP-positive ECs cocultured with GFP-positive BMMCs [BMMCs (+)] displayed an increase of cell density (**B,C**) and of ratio of BrdU-positive cells [RFP (red); GFP (green); BrdU (blue)](**B,D**). (**E**): Endothelial cell proliferation was induced by BMMCs of various densities (1×10^5, 5×10^5, or 1×10^6 cells per well). **(F–H):** BMMCs did not suppress the cell death of ECs in vitro. Compared with ECs without BMMCs [BMMCs (−)][RFP (red); caspase3 (blue)](**F,H**), there is no significant difference in the ratio of RFP/caspase3 double-positive cells to RFP-positive cells in ECs with BMMCs [BMMCs (+)][RFP (red); GFP (green); caspase3 (blue)](**G,H**). **(I):** The supernatant from BMMCs promoted the proliferation of ECs in vitro. Incubation of ECs by BMMCs-CM enhanced the cell density compared with CM. **(J):** ELISA for BMMCs-CM showed an increase of VEGF and IGF-1. **(K, L):** The proliferation of ECs by BMMCs-CM was suppressed by pretreatment for BMMCs-CM with anti-VEGF- (0.01 or 0.1 μg/ml) or IGF-1-neutralizing antibody (0.1 or 1 μg/ml) (**L**). $n = 5$ for each experimental group. $^*p < .05$ versus [BMMCs (−)](**C,D,E**), CM (**I,J**), or BMMCs-CM (**K,L**). Scale bar: 100 μm (**A,F**). Results displayed are representative of five repetitions of the experimental protocol. Abbreviations: BMMCs, bone marrow mononuclear cells; BMMCs-CM, BMMCs-conditioned medium; BrdU, bromodeoxyuridine; CM, control medium; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-basic; GFP, green fluorescent protein; IGF-1, insulin-like growth factor-1; RFP, red fluorescent protein; VEGF, vascular endothelial growth factor.

BMMCs-treated mice displayed improved cortical function (i.e., reduction of locomotion) during the light phase (Fig. 7I). However, improvement of cortical function with BMMCs was significantly suppressed in mice treated with BMMCs plus endostatin (Fig. 7I). Furthermore, we investigated whether locomotion during the light phase is associated with CBF using the Spearman's rank correlation test [PBS groups ($n = 12$), BMMCs groups ($n = 12$), and BMMCs + endostatin groups ($n = 12$); a total of 36 data points], and found that reduction in locomotion during the light phase correlated with an increase in CBF ($r = -0.57$, $p < .05$) (Fig. 7J).

Discussion

This study has demonstrated for the first time that BMMCs promote the proliferation of endogenous NSPCs developing in close proximity to ECs after cerebral infarction through vascular niche regulation, which includes augmentation of ECs resulting from endothelial proliferation (angiogenesis).

We have also shown that transplantation of BMMCs promoted angiogenesis after ischemic stroke as well as after other ischemic diseases such as limb ischemia [16, 17] and

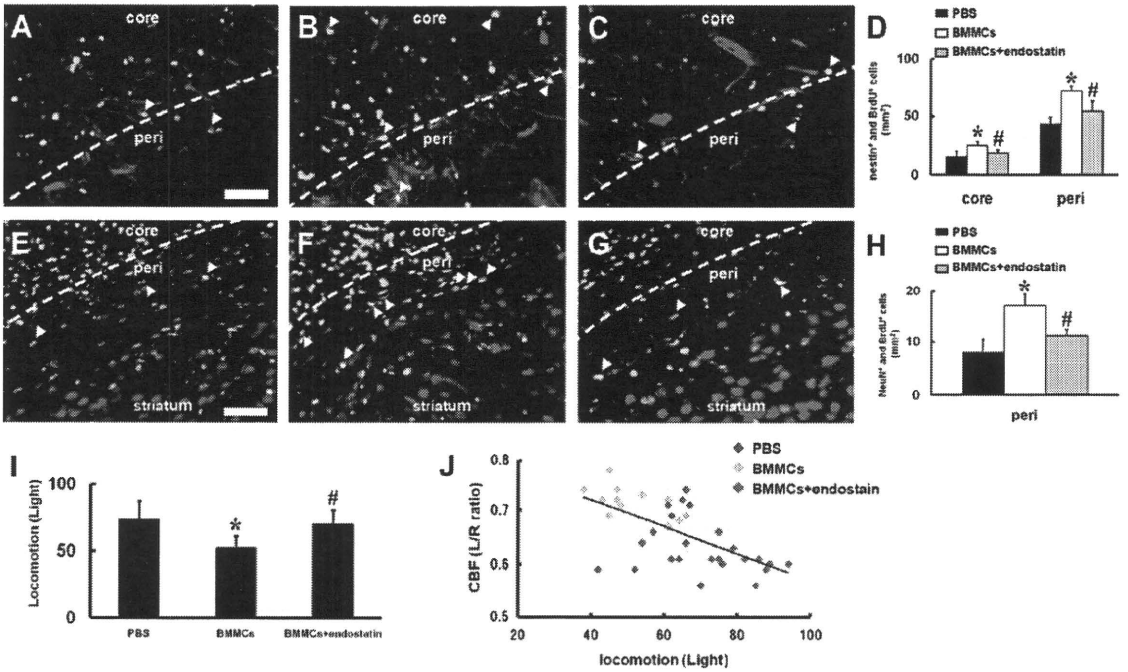

Figure 7. BMMCs promote the proliferation of ischemia-induced neural stem/progenitor cells (NSPCs) and accelerate neurogenesis and functional recovery. **(A–D):** The number of nestin/BrdU double-positive cells was quantified at ischemic core and peri-infarct area on poststroke day 7. Compared with PBS-injected mice [nestin (red); BrdU (green)](**A**, arrowheads; **D**), the number of nestin/BrdU double-positive cells was significantly increased at both regions in BMMCs-treated mice [nestin (red); BrdU (green)](**B**, arrowheads; **D**). However, compared with mice treated with BMMCs alone, additional treatment of endostatin following BMMCs transplantation (BMMCs + endostatin) significantly suppressed the number of nestin/BrdU double-positive cells (**C**, arrowheads; **D**). **(E–H):** The number of NeuN/BrdU double-positive cells was quantified at the peristroke area on poststroke day 30. Compared with PBS-injected mice [NeuN (red); BrdU (green)] (**E**, arrowheads; **H**), the number of NeuN/BrdU double-positive cells was significantly increased in BMMCs-treated mice [NeuN (red); BrdU (green)](**F**, arrowheads; **H**). However, the increase after BMMCs treatment was suppressed by endostatin treatment following BMMCs transplantation (BMMCs + endostatin) (**G**, arrowheads; **H**). **(I,J):** Behavioral analysis was performed on poststroke day 30. Locomotion during the light phase was suppressed in BMMCs-treated mice (**I**). However, improvement of cortical function with BMMCs was significantly blocked in mice treated with BMMCs plus endostatin (**I**). Reduction in locomotion during the light phase was related to an increase in CBF (**J**). $n = 5$ (**D,H**), $n = 12$ (**I**) for each experimental group. $*p < .05$ versus PBS group of each region (**D,H**). $\#p < .05$ versus BMMCs group of each region (**D,H**). $*p < .05$ versus PBS group (**I**). $\#p < .05$ versus BMMCs group (**I**). Scale bar: 100 μm (**A,E**). Results displayed represent five repetitions of the experimental protocol (**A–H**). Abbreviations: BMMCs, bone marrow mononuclear cells; BrdU, bromodeoxyuridine; CBF, cerebral blood flow; core, ischemic core; PBS, phosphate buffered saline; peri, peri-infarct area.

myocardial infarction [18, 19]. In this study, we intravenously transplanted BMMCs and found that most of GFP-positive BMMCs accumulated in and around poststroke region in brains. These findings are consistent with previous findings showing that systemically transplanted BMMCs preferentially homed to ischemic regions [23, 30]. Previous studies have reported that various types of cells including BMCs [45], MSC [26], or HSCs [33] could transdifferentiate into ECs after their transplantation in case of stroke. However, transdifferentiation to cells with endothelial phenotype from GFP-positive cells was only rarely observed (<1%), as was the case in previous reports of cell transplantation by BMCs [45] and MSCs [26]. These observations indicate that BMMCs-induced angiogenesis largely results from the proliferation of endogenous ECs from the adjacent tissue and from circulating endothelial progenitor cells, rather than by angiogenesis derived from grafted BMMCs. Although the mechanisms whereby BMMCs promote angiogenesis in vivo remain unclear, our current coculture experiments indicate that BMMCs-induced proliferation of ECs is at least partially attributable to soluble factors. It has been reported that BMCs such as BMMCs [22, 46, 47] and MSCs [48–50] secrete multiple growth factors, including VEGF, glia-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and hepatocyte growth factor (HGF). In addition, they have been considered to be part of mechanisms responsible for angiogenic, antiapoptotic, and mitogenic effects after cell transplantation [51, 52]. Similar to previous studies, our current study showed that VEGF, which is best known as a key mitogen for ECs [53, 54], could promote the proliferation of ECs in vitro. Interestingly, IGF-1, which had a proangiogenic effect [55], increased noticeably in BMMCs-CM, and it was found that IGF-1 was also related to cell proliferation of ECs in vitro. In the present study, BMMCs-CM induced the proliferation of ECs compared with controls, but maximizing the impact of BMMCs on ECs seemed to be optimal under conditions where the contact between BMMCs and ECs could occur. Thus, although the effect of BMMCs might involve soluble factors, such as VEGF and IGF-1, there seems to be an additional role for cell–cell contact regulation. Furthermore, eNOS expression of ECs was enhanced in BMMCs-treated mice, and nitric oxide (NO) production from eNOS has also been reported to promote neovascularization [56]. Thus, these several factors may contribute to the proliferation of ECs in vivo. The exact mechanisms should be clarified in future studies.

In the present study, nestin-positive NSPCs proliferating in and around the poststroke area were found adjacent to ECs.

These observations may indicate that ischemia-induced NSPCs of the poststroke cortex originate, in part, from microvascular pericytes, as suggested previously [8, 57], although the precise origin of NSPCs is as yet undetermined. Another possibility is that ECs are niches of ischemia-induced NSPCs developing in the cortex of adult brain. It is well-known that in conventional neurogenic regions of the adult brain such as SVZ [58, 59] and SGZ [4], NSPCs reside in vascular niches, and the vasculature is regarded as a key element throughout life. Although the niches for the cortical NSPCs in the adult brain remain unclear, our recent studies [15] and those of others [60, 61] indicate that ECs are likely to be an important element of niches for NSPCs in the cerebral cortex. In support of this viewpoint, we have shown in the current study that proliferation of endogenous ischemia-induced NSPCs was observed more frequently near endogenous ECs in and around the poststroke cortex, and that the grafted ECs promoted the proliferation of endogenous NSPCs in that area. These findings support the hypothesis that ECs are niches for endogenous NSPCs developed in the cerebral cortex after ischemic stroke. In this study, BMMCs promoted the proliferation of ECs in vivo and in vitro, and transplantation of BMMCs could accelerate the proliferation of ischemia-induced NSPCs. However, additional treatment by endostatin following BMMCs transplantation suppressed proliferation of NSPCs by BMMCs. These results strongly suggest that BMMCs promote the proliferation of endogenous ischemia-induced NSPCs through vascular niche regulation, which includes an increase of ECs. However, we cannot completely rule out that BMMCs also directly accelerated the proliferation of the NSPCs, at least in part.

In the present study, BMMCs promoted neurogenesis and functional recovery concomitant with an increase of ECs, including endothelial proliferation (angiogenesis) following ischemic stroke. These observations are consistent with previous studies in which angiogenesis and neurogenesis were accelerated along with neurologic recovery in animal models of stroke after cell transplantation with MSCs [62] and HSCs [33]. In addition, we found that an increase in CBF correlated with reduction in locomotion during the light phase. These observations were consistent with those of previous studies, which showed that increased CBF is associated with improved neurological recovery [63–65]. However, the mechanism of angiogenesis-mediated functional recovery has remained unclear. Although functional recovery is possibly partly attributable to a neuroprotective mechanism of BMMCs as suggested previously [30, 32], our current study showed that additional treatment with endostatin following BMMCs transplantation suppressed the beneficial effects induced by BMMCs, such as proliferation of NSPCs, neurogenesis, and functional recovery. These findings suggest that proliferation of ischemia-induced NSPCs induced by an increase in ECs following BMMCs transplantation might enhance neurogenesis, thereby contributing to functional recovery. These observations are consistent with our recent study, in which cotransplantation of ECs promoted proliferation and neuronal differentiation of grafted ischemia-induced NSPCs with functional recovery [15].

Conclusions

BMMCs can contribute to the proliferation of endogenous ischemia-induced NSPCs developing in close proximity to ECs after cerebral infarction through vascular niche regulation. Our observations provide a novel basic biological mechanism for BMMCs in neurovascular interaction during cortical repair and also suggest that BMMCs transplantation has potential as therapeutic option in stroke treatment.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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