Concurrent Vasculogenesis and Neurogenesis From Adult Neural Stem Cells

Masaaki Ii,* Hiromi Nishimura,* Haruki Sekiguchi, Naosuke Kamei, Ayumi Yokoyama, Miki Horii, Takayuki Asahara

Rationale: Recent reports have demonstrated that signals from vascular endothelial cells are necessary for organogenesis that may precede vasculogenesis. However, the origin of these neovascular cells in regenerating tissue has not been clarified.

Objective: Here we tested the hypothesis that adult neural stem cells (NSCs) can differentiate into vascular lineage, as well as neural lineage, in the process of collaborative organogenesis.

Methods and Results: NSCs, clonally isolated from mouse brain, were shown to develop endothelial and smooth muscle phenotypes in vitro. To elucidate whether NSCs can simultaneously differentiate into vascular and neural cells in vivo, genetically labeled NSCs were administered to mice with unilateral sciatic nerve crush injury or operatively induced brain and myocardial ischemia. Two weeks later, necropsy examination disclosed recruitment of the labeled NSCs to sites of injury differentiating into vascular cells (endothelial cells and vascular smooth muscle cells) and Schwann cells in regenerating nerve. Similarly, NSC-derived vascular cells/astrocytes and endothelial cells were identified in ischemic brain tissue and capillaries in myocardium 2 weeks following transplantation, respectively.

Conclusions: These findings, concurrent vasculogenesis and neurogenesis from a common stem cell, suggest that certain somatic stem cells are capable of differentiating into not only somatic cells of identity but also into vascular cells for tissue regeneration. (Circ Res. 2009;105:860-868.)

Key Words: stem cells ■ ischemia ■ angiogenesis ■ neurogenesis ■ vasculogenesis

Neural stem cells (NSCs) are by definition of self-renewing and classically differentiate into neural lineage cells, including neurons, astrocytes, and oligodendrocytes. Recent reports, however, have demonstrated that NSCs are relatively free from cell lineage restriction compared to other somatic stem cells. NSCs, for example, have been reported to differentiate into endothelial cells, as well as skeletal muscle cells, and blood cells in vitro. Similarly, after coculture with embryonic stem cells, NSCs differentiate into several lineage cells beyond germ lineages when transplanted into an early-stage embryo. The origin of endothelial cells, critical for both blood vessel formation that provides tissue sustenance and possibly organ induction and/or remodeling, has been conventionally assumed to be independent of those cell types that define a given tissue or organ.

Given these precedents and the technical facility with which NSCs may be identified as a clonal stem cell–derived population, we investigated the hypothesis that NSCs may collaboratively differentiate into vascular lineage cells, along with the anticipated neural lineage differentiation not only in vitro but also in vivo involving pathophysiological settings. Such an option would permit NSCs to serve as a source of vascular elements at an early, critical stage of organogenesis in tissue regeneration.

Methods

NSC Isolation

Isolation of NSCs, formation of clonal neurospheres and their characterization were carried out according to previously established methods. The isolated NSCs were passaged suspending at a density of <2500 cells/cm² or <5 cells/mL. These nonclonally isolated NSCs were used at passage 3 for in vivo cell injection study. For clonal experiments, individual cells were transferred with a micropipette to the 96-well microplates and allowed to form neurospheres (Figure 1a). These clonally isolated neurospheres were used for in vitro study. Each neurosphere was then dissociated and expanded. To exclude the possible contamination of vascular

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lineage in the peripheral blood, we isolated NSCs from mice that had received bone marrow transplantation from transgenic mice constitutively overexpressing green fluorescence protein. If bone marrow– derived vascular progenitors are contaminated in the isolated NSCs, we can detect by green fluorescence protein. The obtained neurospheres were not contaminated with BM-derived cells (data not shown). This result rules out the possibility of the contamination with vascular progenitors.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and describes all other methods and materials used in this study.

**Results**

**Characterization of Neurosphere**

The expression of nestin, a marker for neural stem/progenitor cells,11 was detected in neurospheres (Figure 1a and 1b). Neurospheres continued their growth by repeated cell divisions until reaching a radius of approximately 250 to 350 μm, typically within 3 weeks. Why most of the neurospheres cease growing after reaching a radius of 250 to 350 μm is unknown; this distance is, however, coincident with the maximum distance of oxygen diffusion in normal or malignant tissue.12,13 It is thus possible that cessation of neurosphere growth results from nutrient diffusion distance from the surface to the core area of the neurospheres, rendering the core areas relatively hypoxic. To test this hypothesis, we examined the expression of hypoxia inducible factor (HIF)-α and vascular endothelial growth factor (VEGF), typically induced by hypoxia.14,15 However, immunostaining revealed that both small and large neurospheres that ceased growing expressed HIF-1α (Figure 1c) and abundant VEGF protein (Figure 1d) uniformly in spheres as well as nestin, suggesting that the cells in the inner and outer mass of a large sphere show similar phenotype in terms of VEGF, nestin, and Hif-1α distribution pattern. To further clarify the relationship between the size of neurospheres, neurospheres were separated into 2 types including small neurospheres with a radius of less than 100 μm and large neurospheres with a radius of more than 300 μm, and phenotypic difference of these neurospheres and dissociated NSCs were assessed by quantitative real-time RT-PCR for hypoxia-inducible and angiogenic molecules. HIF-1α, VEGF, and angiopoietin-2 (Ang-2) mRNA expressions were high and Ang-1, platelet-derived growth factor-B (PDGF-B), and nerve growth factor (NGF) mRNA expressions were low in both small and large neurospheres. Interestingly, only Hif-1α and Ang-2 mRNA expressions were significantly downregulated in dissociated NSCs (Figure 1e). The induction/regulation pattern of Hif-1α seen in large neurospheres is typical of hypoxic tissues and promotes vascular formation.16 Expression of AC133 mRNA, conventionally viewed as a common marker for hematopoietic stem, endothelial progenitor, and neural stem cells,17–20 was also detected in both small and large neurospheres and dissociated NSCs regardless of the original size of neurosphere (Figure 1e).

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>Ang</td>
<td>Angiopoietin</td>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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<tr>
<td>BS</td>
<td>Bandeiraea simplicifolia</td>
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<td>Dil</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NRP</td>
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<td>SM</td>
<td>Smooth muscle</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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**Figure 1.** Characterization of neurospheres by the expressions of VEGF, Ang 2, PDGF-B, and AC133. a, Phase-contrast photomicrograph of a neurosphere on day 14 after isolation. Immunostaining of both small and large neurosphere for nestin (b), Hif-1α (c), and VEGF (d). Scale bars=100 μm. e, Gene expressions in neurospheres and dissociated neurosphere-derived NSCs. Small and large neurospheres were dissociated into single NSCs and cultured for 6 to 8 hours on noncoated dishes with NSC expansion medium. The dissociated NSCs and neurospheres were examined by real-time RT-PCR. Perventricular forebrain tissue was used as a reference control. Each gene expression was expressed as a relative mRNA expression normalized to GAPDH, and more than 50 is considered to be a significant expression as transcripts. The experiment was performed in triplicate, and RNA extracted from 3 samples was analyzed.
Figure 2. Analysis of vascular SM and endothelial lineage features of attached colony derived from neurospheres. a, Expression of SM \(\alpha\)-actin in large neurospheres. Immunostaining (red) (left) and Northern analysis (right) documents that large neurospheres express SM \(\alpha\)-actin. Scale bar: 100 \(\mu\)m. Total RNA from lung tissue was also loaded for positive control. b, Staining of large neurosphere with calponin (red). Scale bar: 100 \(\mu\)m. Boxed area is magnified. c, An in vitro endothelial differentiation assay was used. Large neurospheres were cultured on poly-L-ornithine (PLO)/laminin-coated dish with NSC culture medium for the first 2 weeks and with endothelial differentiating medium for the following 2 weeks. The colony from an attached large neurosphere was first incubated with DiI-labeled acetylated low-density lipoprotein (AcLDL) (red) and then reacted with FITC-BS lectin (green) and DAPI (blue). Scale bars: 200 and 50 \(\mu\)m for upper and lower images, respectively. d, Staining of attached large neurospheres with isolectin B4 (red). Boxed area is magnified. Scale bar: 200 \(\mu\)m. e, RT-PCR analysis for Flk-1 mRNA induction in attached larger neurospheres after culturing with vascular cell-oriented medium. cDNA from lungs was used for the positive control. f, Phase-contrast pictures of attached large neurospheres cultured...
These findings may reflect the demand and potential within the neurosphere for both neural and vascular development.

**NSC Differentiation Into Vascular and Neural Lineage In Vitro**

To elucidate whether NSCs have an ability to differentiate into vascular cells in vitro, markers for vascular smooth muscle (SM) and endothelial cells were examined. Protein and mRNA expression of vascular SM α-actin and calponin, considered as vascular SM cell markers, were detected in unstimulated large neurospheres as well (Figure 2a and 2b). When large neurospheres were cultured on poly-L-ornithine/laminin coated dishes with NSC culture medium for the first 2 weeks and another 2 weeks with vascular cell–orientated medium, the cells of the spreading colony took up 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated low-density lipoprotein and were stained positively for fluorescein isothiocyanate (FITC)-labeled *Griffonia simplicifolia* lectin I (BS lectin) (Figure 2c). Large neurospheres cultured on Matrigel (BD) under same culture conditions were also shown to be positive for isolecithin B4 (Figure 2d). RT-PCR disclosed Flk-1 induction in the attached neurospheres in a time dependent manner (Figure 2e). These features represent characteristics of endothelial lineage in large neurospheres. Moreover, the attached large neurosphere cultured for further 1 week with vascular cell–orientated medium formed tube-like structure in network (Figure 2f, arrows), as well as neuron-like cells (Figure 2f, arrowheads), and the gene expression levels in neurospheres before and after vascular differentiation assay were examined by real-time RT-PCR. The expressions of mRNA for endothelial (CD31, Tie-2, and endothelial nitric oxide synthase) or vascular SM cell (SM α-actin, calponin, and PDGF receptor (PDGFR)-β) lineage markers were significantly upregulated by culture with vascular cell–orientated medium both in small and in large neurospheres except for CD31 and Tie-2 expressions in small neurospheres. On the other hand, expressions of mRNA for glial fibrillary acid protein (astrocyte-specific marker) were strikingly upregulated, whereas nestin (neural stem cell marker) and microtubule-associated protein (MAP)-2 (neuron-specific marker) were dramatically downregulated both in small and in large neurospheres. The mRNA expression levels of vascular cell marker, endothelial nitric oxide synthase, SM α-actin, calponin, and PDGFR-β in differentiated small neurospheres were lower than those in large neurospheres (Figure 2g). Taken together, these results indicate that clonally derived neurospheres/NSCs have a potential of simultaneous differentiation into the vascular and neural lineages.

**Expression of Interactive Cell–Cell Signaling Molecules in NSC-Derived Vascular Lineage Cells**

In the process of neurogenesis, microenvironmental interaction with vascular endothelial cells plays a pivotal role. Not only respective paracrine factors for neurogenesis and vasculogenesis, such as NGF and VEGF, but also interactive cell–cell contact signals via membrane-bound ligand-receptor systems, eg, Notch-Delta/Jagged and ephrin–Eph system, should closely regulate both regenerative cascades. The clonally obtained secondary small neurospheres were cultured under the condition for neural differentiation in the presence of VEGF or NGF with or without the neutralizing antibody. After 8 hours, RNA was isolated and RT-PCR analysis for notch and ephrin systems was carried out. Jagged-1 is expressed in endothelial and SM lineage, as well as neural cells, and ephrin B2 is also expressed in (arterial) endothelial lineage, as well as certain particular neural cell types, such as dopaminergic neurons in midbrain and astrocytes in subventricular zone. These genes were clearly detected by RT-PCR analysis (Figure 3, lane 3). Furthermore, VEGF upregulated endothelial-contacting ligands, Jagged-2 and ephrin B2 expressions, (Figure 3, lane 4) and blockage of VEGF cancelled these gene expressions (Figure 3, lane 5). On the other hand, stimulation of neurospheres by NGF resulted in the indicated gene expression pattern (Figure 3, lane 6), which is similar to that with vehicle treatment (Figure 3, lane 3) and with VEGF-neutralizing medium (Figure 3, lane 5). The blockade of NGF signaling disclosed the upregulation of not only Jagged-2 but also Delta-like 4 genes, which are endothelial-specific Notch ligands (Figure 3, lane 7). Interestingly, Notch 1 and Eph B4, interactive receptors for Jagged-1 and -2 and Delta-like 4 and ephrin B2, respectively, are expressed in small neurospheres regardless of culture conditions (Figure 3, lanes 2 to 7).

We further examined receptor gene expressions for VEGF and NGF in small and large neurospheres to clarify which type of receptor plays a critical role in response to the ligands by quantitative real-time RT-PCR. Interestingly, only neuropilin (NRP)-1 mRNA expression in both small and large neurospheres was significantly high among VEGF coreceptors, suggesting that NRP-1 might be a responsible receptor among for upregulation of interactive vascular cell–cell contact–related gene expressions by VEGF stimulation. However, no significant expressions of representative NGF receptors, p75 and TrkA, were detected in both small and large neurospheres (Online Figure I). These findings suggest the following possibilities. In neurospheres, (1) NRP-1 may also be another responsible receptor of NGF rather than p75 or TrkA. (2) Because NRP-1 is a receptor subunit of the extracellular molecule semaphorin 3A, which closely interacts with NGF signaling, NGF signaling might be indirectly activated via sema-
phorin 3A/NRP-1 binding. (3) Because NGF upregulates VEGF and its receptor expressions including NRP-1 in certain cell types, VEGF might mediate NGF signaling. Taken together, these results indicate that newly generated neural and vascular lineage cells from NSCs express specific cell–cell interactive signaling systems, which are possible to communicate for collaborative signaling for organogenesis.

**NSC Differentiation Into Vascular and Neural Lineage in Injured Nerve**

To explore whether NSCs can differentiate into vascular cells in vivo, a nerve crush injury experiment was used as the simplest model to detect vasculogenesis in nervous system. NSCs were isolated from Rosa 26 mice in which all cells constitutively express β-galactosidase (β-gal) and expanded ex vivo by forming neurospheres. Unstimulated neurospheres, which did not express CD31, Tie-2, or Flk-1, as shown in Figure 2c and 2g, were dissociated into NSCs to inject. After unilateral sciatic nerve crush injury, the dissociated NSCs were administrated via a tail vein. Before recovery of nerve conduction velocity, robust angiogenesis is typically seen within the nerve tissue. Whereas no evidence of neovascularization was observed in the control (noninjured) nerve, robust angiogenesis was observed in the crushed nerve (Figure 4a). Whole mount staining of sciatic nerves demonstrated that these foci of neovascularization, identified by immunopositivity of FITC-conjugated BS lectin, which was infused systemically just before euthanasia of the animals, coexpressed β-gal, indicating contributions of transplanted NSCs in the vascular components (Figure 4b). These NSC-derived blood vessels occupied around 8% of those of newly formed vasculatures in the nerve regenerating tissues (data not shown). Immunostaining of frozen sections also revealed that a certain amount of endothelial cells and SM cells within foci of neovascular formation, as well as Schwann cells, stained positively for β-gal (Figure 5).

We also examined NSC homing to major organs as well as injured sciatic nerve. DiI-labeled NSCs (10^7/mouse) were systemically injected to mice immediate after surgery and the DiI (red fluorescent) positive area (NSCs) was observed on back surface under fluorescent dissection microscope 2 weeks after cell injection. Remarkable red fluorescent signal was detected at the site of injured sciatic nerve, whereas little red signal was detected on the other sites, suggesting that a number of injected DiI positive NSCs were accumulated in the injured sciatic nerve rather than intact other organs (Online Figure II). Consistent with the result of DiI detection in whole body, histological analysis exhibited a certain number of recruited DiI-NSCs to intact organs such as brain, lung, kidney, liver, and heart (data not shown).

We further examined the therapeutic effect of NSCs on functional recovery in injured nerve. Systemic NSC transfu-
planted NSCs were demonstrated by immunostaining for S-100 and glial fibrillary acid protein, which were identified by the expression of glial fibrillary acid protein, was also detected around the β-gal–expressing vascular structures. There were, however, no β-gal–expressing neurons, identified by the MAP-2 immunostaining (Figure 6a).

**NSC Differentiation Into Endothelial Lineage in Ischemic Myocardium**

Finally, to determine whether NSCs can differentiate into vascular cells in response to a nonneural pathological insult, NSCs were isolated from Rosa 26 mice and expanded ex vivo by forming neurospheres. Dissociated NSCs were injected via a tail vein into C57B6/J mice with myocardial infarction induced by coronary artery ligation. Immunohistochemical analysis using an antibody directed against β-gal revealed that β-gal–positive cells (red fluorescent) expressed endothelial-like morphology and immunopositivity for FITC-conjugated BS lectin (green fluorescent), which was infused systemically just before euthanasia of the animals, (Figure 6b) suggesting that NSCs recruited to neovascular foci with the endothelial cell characteristics. The finding was more evident in ischemic myocardium 2 weeks (Figure 6b, lower) than 1 week (Figure 6b, upper) after surgery. Expression of β-gal in intact myocardium was observed only in rare cells; none was detected in vehicle-treated mice (data not shown). However, some of the injected NSCs surprisingly recruited to nonischemic intact capillaries in liver that was isolated from the mice underwent myocardial infarction surgery (Figure 6c). These findings thus demonstrate that NSCs can differentiate into vascular cells in the setting of nonneural tissue ischemia or even in nonischemic tissue.

**Discussion**

In the present study, we have shown the following series of evidences: (1) triple characteristics of neural, endothelial, and SM progenitors in clonally isolated NSC-derived neurosphere; (2) differentiation capacity of the expanded neurosphere/NSCs into neural, endothelial, and SM lineages; (3) neural- or vascular growth factor–dependent NSC differentiation into functional vascular lineage cells involving specific interactive cell–cell signaling; and (4) in vivo NSC contribution to both neurogenesis and vasculogenesis in not only neuronal tissue but also nonneural tissue in adults.

Our results established that NSCs retain the ability in adult mammals to differentiate into endothelial and SM lineage cells along with neural lineage cell differentiation. Vascular cell–like phenotypes with the expressions of Notch signaling molecule in neurospheres were induced under certain culture conditions such as stimulation of VEGF signaling or blocking NGF signaling. Particularly, the blocking of NGF signaling in neurospheres resulted in the induction of more endothelial

**Figure 5. Differentiation of NSCs into endothelial cells, vascular SM cells and Schwann cells in crushed nerves.** NSCs from Rosa 26 mice were administered to mice with sciatic nerve crush injury. After 2 weeks, the sciatic nerve was dissected and frozen sections were made. Endothelial cells and vascular SM cells were identified by isolectin B4 (ILB4) (left) and SM α-actin (middle), respectively. Vasculature and cells derived from transplanted NSCs were demonstrated by immunostaining for β-gal. Arrows in the left and middle columns indicate double-positive vasculature. Arrowheads in the left and middle columns denote ILB4 or SM α-actin positive but β-gal-negative portions, inferred to derive from original host cells. Schwann cells (right column) were identified by S-100 antibody. Arrows in the right column indicate cells that are double-positive for S-100 and β-gal. Boxed areas in the top photomicrographs are magnified in the lower photomicrographs of each column. Scale bars: 200 μm (black); 50 μm (white).
specific gene expressions involving Delta-like 4 ligand than the stimulation of VEGF signaling, suggesting that because neurospheres themselves produce both VEGF and NGF (Figure 1), and these growth factors are thought to play a role in neurospheres as autocrine factors for the growth/differentiation, suppression of NGF-involved neuronal signaling pathways would be more critical for their differentiation into vascular lineage cells rather than more than stimulation of VEGF signaling. These findings may give rise to a novel mechanistic insight that the direction of NSC differentiation toward vascular or neuronal lineage is determined depending on the balance of exposure to angiogenic and neurogenic factors. Indeed, transplanted NSCs differentiated into vascular cells not only in neural tissues but also nonneural tissues in vivo. However, the differentiation of recruited NSCs into neuronal cells could not be detected immunohistologically (data not shown) in ischemic myocardium and nonischemic liver (Figure 6b). These findings suggest that the microenvironments, which are not always specific for the nervous system, are important in vascular differentiation from NSCs. Also, because undifferentiated neurospheres exhibit distinct expression patterns of Notch ligands and Eph B4 as well as both vascular and neuronal markers, cell–cell interaction through Notch signaling or Eph/ephrin signaling might also play a crucial role in vascular differentiation from NSCs.

Consistent with the observation in a previous study that adult neurogenesis occurs within an angiogenic niche where active vascular recruitment occurs,26 our findings indicate that foci of neurogenesis and vasculogenesis within a given cluster may derive from common stem or progenitor cells. Transplanted NSCs practically contributed to vasculogenesis as well as gliogenesis both in damaged peripheral and central nervous system in vivo. Unexpectedly, no neurons derived from exogenously transplanted NSCs were observed in infarct brain in our hands, whereas transplanted NSC-derived vascular cells and astrocytes were observed. Although the differentiation potential of adult NSCs into neurons is quite limited in vitro (<1%),24 NSCs in adult brain stem cell niches in subventricular zone and hippocampus evidently provide neurons during homeostasis and regeneration.33,34 The discrepancy between our observation and previous reports may be attributable to the reason why. (1) NSCs were transplanted at acute injured tissues and influenced by inflammatory stimuli for not neuronal but glial lineage induction. (2) NSCs
derived from different portion from specific lesion of subventricular zone or hippocampus in brain, and (3) ex vivo culture expanded NSCs were used in our study.

Our findings not only add to the multipotent repertoire established previously for NSCs but also provide in vitro and in vivo evidences for concurrent vasculogenesis and neurogenesis from common stem cells, which may contribute to reparative organogenesis. However, because not only neurospheres but also other spheres, ie, cardiospheres, are highly motile and prone to fuse, it is difficult to determine whether the spheres are clonal or oligoclonal in nature. Moreover, the multipotentiaility of a single NSC cannot be assessed, and the possibility exists that cells in the spheres are unipotent, bipotent, or truly multipotent even though clonally isolated NSCs were examined in the experiments. In addition, based on recent evidences in which close correlation between neurogenesis and vascular niche in the subventricular zone has been shown, we speculate that vascular niche is required to induce neurogenesis from adult NSCs. Other reports also demonstrate that endothelial cells play a critical role in the early stage of liver organogenesis or pancreatic differentiation, before blood vessel function. The origin of vascular cells in forming a tissue or organ from stem cells, however, has not been identified completely. Angiogenesis and vasculogenesis are currently classified by the origin of preexisting mature endothelial cells and bone marrow–derived endothelial progenitor cells in adult organs.

In this study, we have shown concurrent tissue regeneration from common stem cells and therapeutic potential of NSCs for neuronal tissue damage. Interestingly, although the favorable effect of NSCs on stroke is reported, there was no therapeutic effect of NSC transplantation on myocardial infarction in our hands (data not shown), suggesting that NSCs have a therapeutic effect on neuronal tissue damage including mechanical injury and ischemia but not on nonneuronal tissue ischemia. The differential effects of NSCs on certain diseases might depend on different tissue regeneration process following injury. In addition, because not only in injured nerve but also ectopic NSC recruitment was observed when NSCs were infused systemically, local transplantation of NSCs might be more safe with better outcome as a therapeutic application. This vasculogenesis from somatic NSCs may be the third component of vascular formation in adult tissue regeneration specifically in neuronal tissue.

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Disclosures
None.

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Cell cultures.
After exanguination via the cardiac apex to minimize contamination of brain tissue with peripheral blood, isolation of NSCs was carried out by previously established methods\(^1\) using 4-6 week-old C57BL/6J or B6.129S7-Gt(ROSA)26Sor/J (Rosa 26, Jackson) mice. The obtained cells were suspended at a density of 1000-2500 cells/cm\(^2\) or < 5 cells/ml. For clonal experiments, individual cells were transferred with a micropipette to the microwells of 96-well plates and allowed to form neurospheres. Each neurosphere was then dissociated and expanded. The NSC culture medium consisted of 20 ng/ml EGF (BectonDickinson), 20 ng/ml bFGF (Upstate Biotechnology), B27 supplement (Life Technologies), 2 mM glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin in DMEM/F12 (Life Technologies). Growth factors were added every other day and half the volume of the medium was changed every fourth day. EGM-2-MV BulletKit (Clonetics) was also used as vascular cell-orientated medium.

RT-PCR and quantitative real-time RT-PCR.
Total RNA was extracted from a single neurosphere or an attached colony derived from a single neurosphere with the RNAqueous kit (Ambion). After DNase I (Ambion) treatment, reverse transcription and PCR were performed using a Superscript Preamplification System Kit (Life Technologies) and Advantage cDNA Polymerase Mix (Clontech), respectively. The denaturing step was 95°C for 30 seconds; the annealing and extension was 64°C for 3 minutes. After repeating 30 to 35 cycles, a prolonged extension step was carried out for 4 minutes. The amplified DNA was electrophoresed in 1.5 % agarose gel containing ethidium bromide and photographed under a UV transilluminator. For quantitative RT-PCR, the converted cDNA samples (2 \(\mu\)l) were amplified in triplicate by real-time PCR machine (ABI Prism 7700, Applied Biosystems) in a final volume of 20 \(\mu\)l using SYBR Green Master Mix reagent (Applied Biosystems) with gene-specific primers. Melting curve analysis was performed with Dissociation Curves software (Applied Biosystems) and the mean cycle threshold (Ct) values were used to calculate gene expressions with normalization to rat GAPDH (rGAPDH).

Northern-blot analysis.
Total RNA was extracted from neurospheres or lung tissue with the RNeasy kit (Qiagen). For Northern blots, 20 mg of total RNA was electrophoresed and transferred to a nylon membrane. Blots were hybridised with a 32P-labeled SM \(\alpha\)-actin probe, washed and exposed to X-ray film.
Animal studies.  
For animal studies, all experimental procedures were conducted in accordance with the Japanese Physiological Society Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the Ethical Committee in Institute of Biomedical Research and Innovation (IBRI) and RIKEN Center for Developmental Biology.

Nerve crush injury model.  
The sciatic nerves of 8-10 week-old male C57BL/6J mice (Jackson) were subjected to unilateral nerve crush injury. Mice were anesthetized with 250mg/kg i.p. of 2, 2, 2-tribromoethanol (Avertin\textsuperscript{TR}, Sigma). The sciatic nerve was exposed after surgical incision of the overlying skin and muscles, and the nerve was crushed at mid-thigh level for 10 seconds, using a hemostat with tips covered by plastic tubing. The skin was closed with a surgical stapler. Mice were sacrificed at 2 weeks post-crush injury.

Neurophysiological measurements.  
Sciatic nerve conduction velocity was measured using standard orthodromic surface recording techniques and a Teca TD-10 (Oxford Instruments) portable recording system in all mice at baseline (before treatment) and then at 2 weeks and 4 weeks after treatment as described previously.\textsuperscript{2} Briefly, motor nerve conduction velocity (MCV) was calculated by dividing the distance between stimulating electrodes by the average latency difference between the peaks of the compound muscle action potentials (CMAPs) evoked from two sites (sciatic notch and ankle). Sensory nerve conduction velocity (SCV) was calculated by dividing the distance between stimulating and recording electrodes by the latency of the signal from the stimulation artifact to the onset of the peak signal. For each nerve, maximal velocities were determined bilaterally. All measured data from both sides are averaged.

Sublethal Forebrain Ischemia Model.  
C57Black/Crj6 mice (Jackson), age 8-10 week-old male, were used to make the sublethal brain ischemic models as described previously.\textsuperscript{3} Under anesthetization with 250mg/kg i.p. of 2, 2, 2-tribromoethanol (Avertin\textsuperscript{TR}, Sigma), bilateral common carotid arteries were isolated and clamped by hemostatic clips for 18 min. The skin was closed with a surgical stapler. Mice were sacrificed at 14 days post-ischemia.

Myocardial Infarction Model.  
C57BL/6J mice (Jackson), age 8-10 weeks old, were anesthetized with 250mg/kg i.p. 2, 2, 2-tribromoethanol (Avertin\textsuperscript{TR}, Sigma) and underwent surgery for myocardial infarction as
described previously. After induction of anesthesia, the mice are placed in a supine position and intubated with a 22G IV catheter (Johnson & Johnson). Respiration is controlled by mechanical ventilation using a rodent ventilator (Harvard Apparatus) (tidal volume 0.3ml, rate $10^5$ strokes/min). Using a dissecting microscope (Olympus SZH 10), a left thoracotomy is performed in the fourth intercostal space. The medial aspect of the incision is extended cranially to form a flap that was retracted to expose the heart. A small opening is made in the pericardium and a 7-0 monofilament nylon suture on a curved taper needle is passed under the left anterior descending artery (LAD) just proximal to the first diagonal branch. Infarction was induced by LAD permanent ligation with the suture. The procedure is completed after closing the chest and eliminating thoracic air.

**In Situ Fluorescent Staining.**
Vascularity of tissues (sciatic nerve, heart, and liver) was examined by in situ fluorescent staining using an endothelial cell-specific marker FITC conjugated BS1-lectin (Vector Laboratories) as described previously. After anesthesia, BS1-lectin (0.1mg per mouse) was injected to mice systemically via a left ventricle of the heart. Ten minutes later, the mice were sacrificed and tissues were harvested and fixed in 4% paraformaldehyde for 2 hours. After fixation, specimens were embedded in OCT compound for frozen cross-section or examined directly for whole mount sciatic nerve staining under computer-assisted fluorescent microscope. (Nikon)

**Immunocytostaining and Immunohistochemistry.**
Neurospheres were preserved in 2% paraformaldehyde in PBS for 10 min and then placed in HistoGel (Richard-Allan Scientific) before embedding in paraffin. In the nerve crush model, isolated sciatic nerves for whole mount staining were fixed by 2% paraformaldehyde for 10 min. For frozen sections, sciatic nerves and forebrains were placed in OCT compound (Sakura Finetechinical) and frozen by liquid nitrogen. Immunostaining of neurospheres was performed using antibodies to nestin (1:300, PharMingen), VEGF (1:100, PharMingen), HIF-1a (1:500, Sigma), SM $\alpha$-actin (1:300, Sigma), calponin (1:300, DAKO), bacterial $\beta$-galactosidase (1:500, Cortex Biochem), CD31 (1:300, PharMingen), S-100 (1:400, PharMingen), GFAP (1:300, SantaCruz) and MAP-2 (1:300, SantaCruz). Chemical staining using FITC-conjugated isoelectin B4 (Vector Laboratories), or DIL-labeled acLDL (Biomedical Technologies) was performed as previously described. After staining, samples were viewed with an inverted fluorescent microscope (Nikon).
Statistical Analysis.

All values were expressed as mean ± SEM. Statistical analyses were performed with commercially available software (GraphPad Prism™, MDF software, Inc.). Comparisons between multiple groups and two groups were tested for significance via analysis of variance (ANOVA) followed by post-hoc testing with the Tukey procedure and nonparametric Mann-Whitney test, respectively. A $P$ value less than 0.05 was considered significant.

References


Online Figure I. VEGF and NGF receptor gene expressions in neurospheres. Small and large neurospheres were examined for the indicated VEGF and NGF co-receptor mRNA expressions by real-time RT-PCR. Brain tissue was used as a control. Each gene expression was expressed as a relative mRNA expression normalized to GAPDH, and over 50 is considered to be a significant expression as transcripts. NRP-1/-2: neuropilin-1/-2. The experiment was performed in triplicate, and RNA extracted from three samples was analyzed.
Online Figure II. Macroscopic red fluorescent detection following systemic DiI labeled NSC injection in whole mouse. 

a, DiI labeled NSCs were systemically injected to the mouse with right sciatic nerve crush injury. DiI positive NSC homing to major organs was assessed by fluorescent imaging system 2 weeks after cell injection. The whole back skin was removed to avoid capturing non-specific fluorescent signals, and NSC recruitment to tissue was recognized as red fluorescent signals on the back of the mouse. Arrow indicates the injured site with high red signal. Bar = 10 mm.

b, Sciatic nerve with muscle, kidney, lung, brain, liver, and heart were harvested from the mouse and observed. Arrows indicate red signals that correspond to DiI labeled NSC recruited sites. Bar = 3 mm.
Online Figure III. Systemic NSC transplantation improved functional recovery in injured nerve. Either NSCs ($1 \times 10^5$/mouse) or PBS (control) were injected to the mice with crushed nerve via a tail vein. To evaluate functional recovery in injured nerve, motor nerve conduction velocity (a) and action potential (b) were measured before (pre), immediately (post), 14, 21, 28 days after sciatic nerve crush injury. *, $P < 0.05$ and **, $P < 0.01$ vs. PBS, n=4 in each group.