(n=3) (d) and flow cytometry to detect Sca-$1^{(+)}$/ Flk-$1^{(+)}$ cells (n=3) (e). BM-KSL subpopulation were expanded for 7 days, then subjected to flow cytometry or the EPC-CFA. EPC conforming cells from total BM were recalculated (n=4). *, P<0.05 ; ***, P<0.001.

Online Figure V: Gene expression of angiogenic cytokines in response to ischemia. RNA expression of VEGF, angiopoietin and tie2 was drastically altered in KSL CD34 subpopulations of lnk deficient mice.

Online Figure VI: BM putative EPCs-derived capillaries in animals receiving Lnk-deficient EPCs. Relative ratio of BM-derived putative EPCs (*i.e.*, $GFP^{(+)}$/ $IB4^{(+)}$ cells) by capillary cells ($IB4^{(+)}$ cells) in mice undergoing BMT (n=6 in each group). **, P<0.01.

Online Figure VII: Regeneration of astrocyte network at P17 retina. (a, b) Representative double staining for rhodamine-conjugated Con A (red, EC marker) and GFAP (green, astrocyte marker) at P17 in Lnk-deficient and WT mice. Mature astrocyte network spreading along the well developed vasculature was observed in *lnk* $^{-/-}$ mice, whereas immature (abnormal) astrocyte network in the avascular lesion was identified in WT. (c) Percent abnormal astrocyte network area in Lnk-deficient and WT mice.

Online Figure VIII: Non-pathogenic effect of retinal vascular disease in *lnk*$^{-/-}$ mice.

(a) Representative HE staining at P17 in *lnk*$^{-/-}$ and WT mice. Arrows indicate pathogenic retinal hemorrhages, which are observed in WT but not in *lnk*$^{-/-}$ mice.
(b) Number of hemorrhagic legions at P17 in retinas of *lnk*$^{-/-}$ and WT mice (n=8).

Online Figure IX: Retinal hemorrhage at P17 retina. Representative images of retinal hemorrhage, a sign of pathogenic angiogenesis, in *lnk* $^{-/-}$ and WT mice at P17.

Online Figure X: Gene expression of angiogenic cytokines in cultured EPCs under hypoxic condition. Quantitative RT-PCR analysis revealed significant upregulation of VEGF, Ang-1 and eNOS, in *lnk*$^{-/-}$ EPCs compared with WT EPCs

(n=8 in each group).

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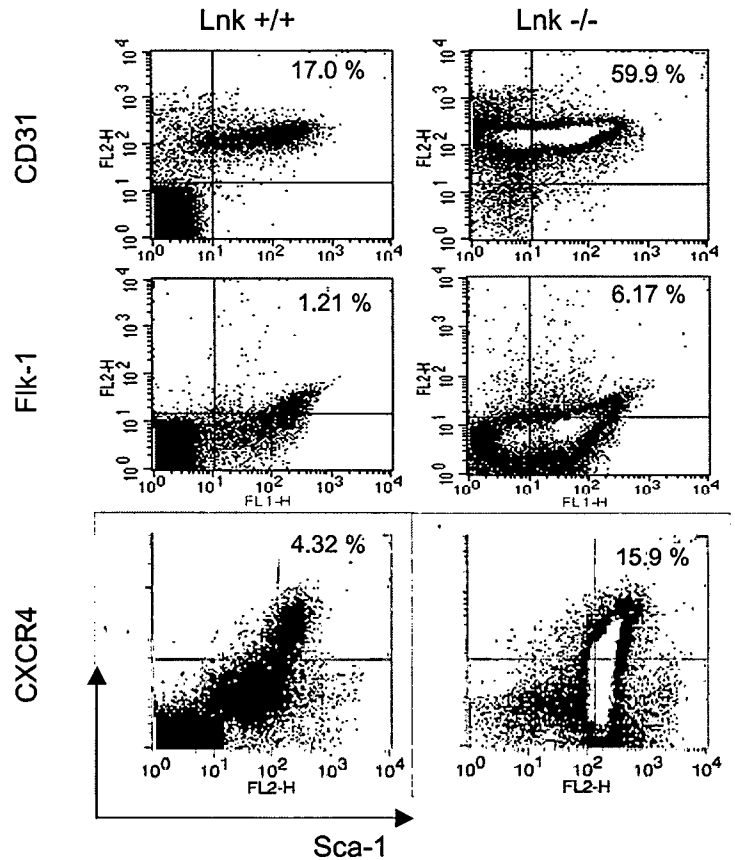

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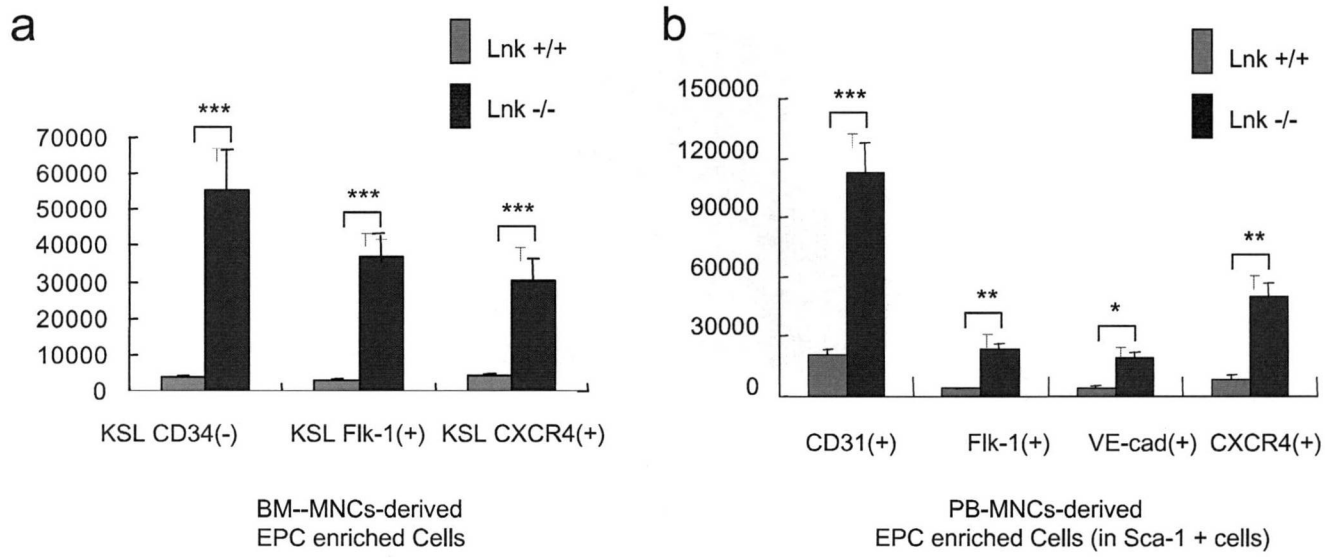

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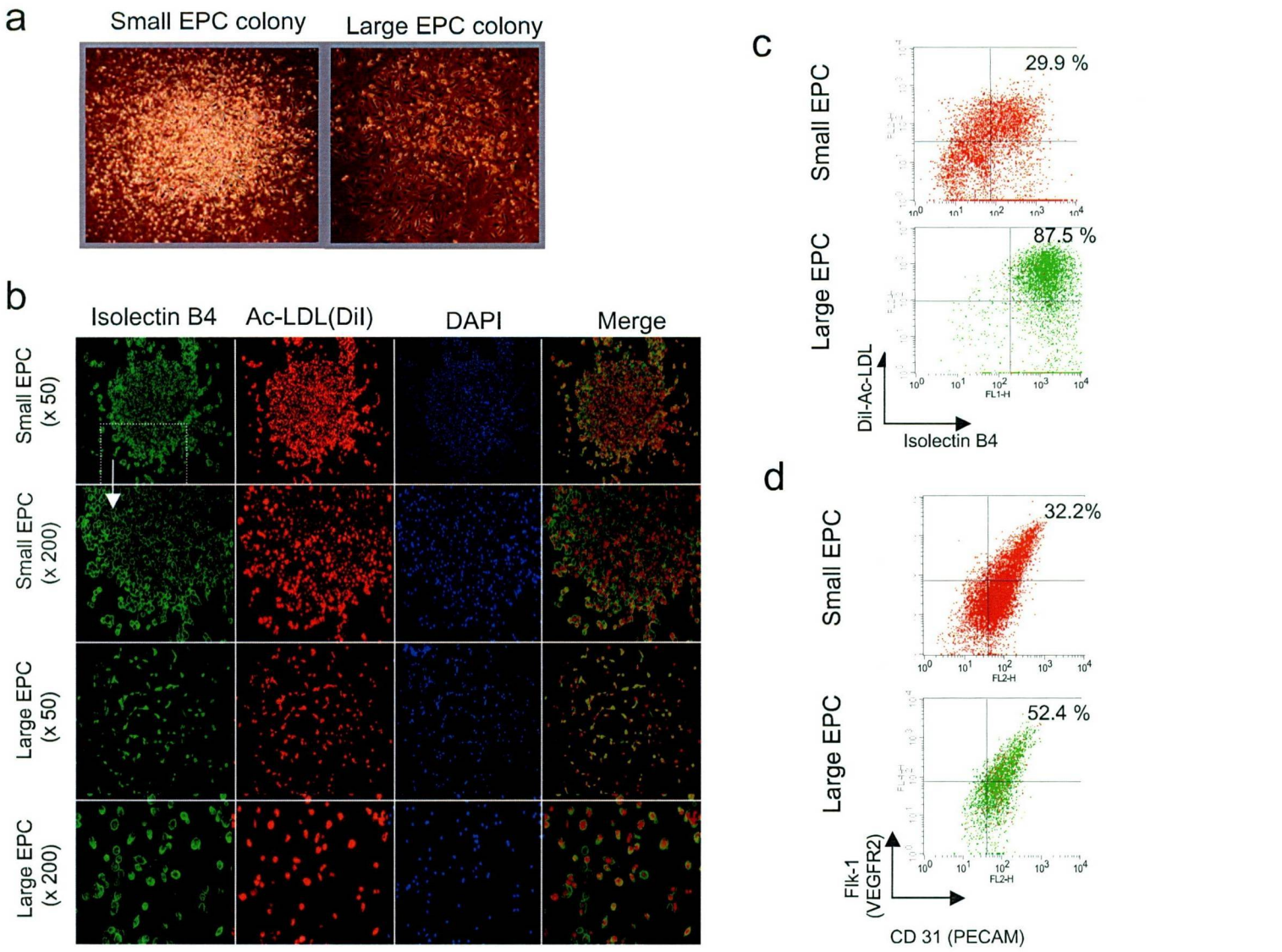

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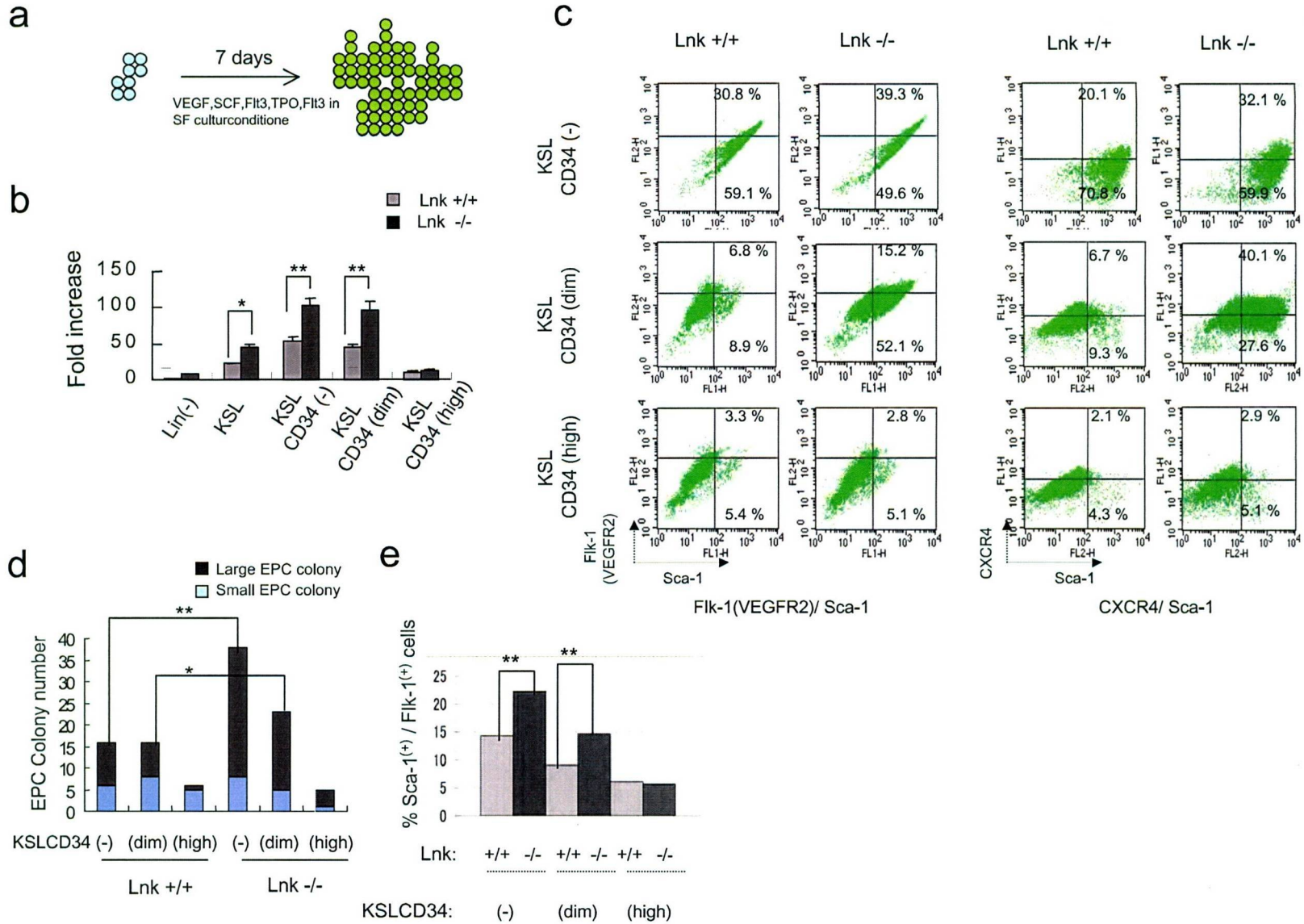

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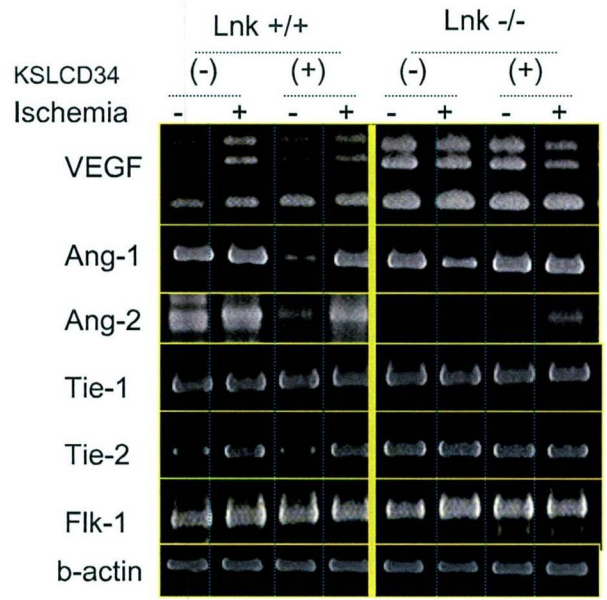

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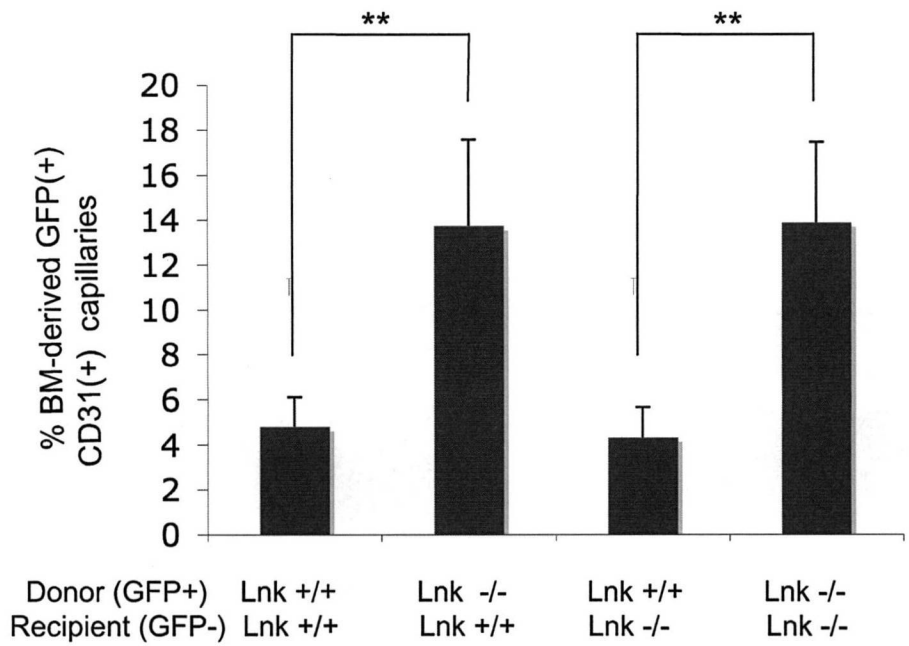

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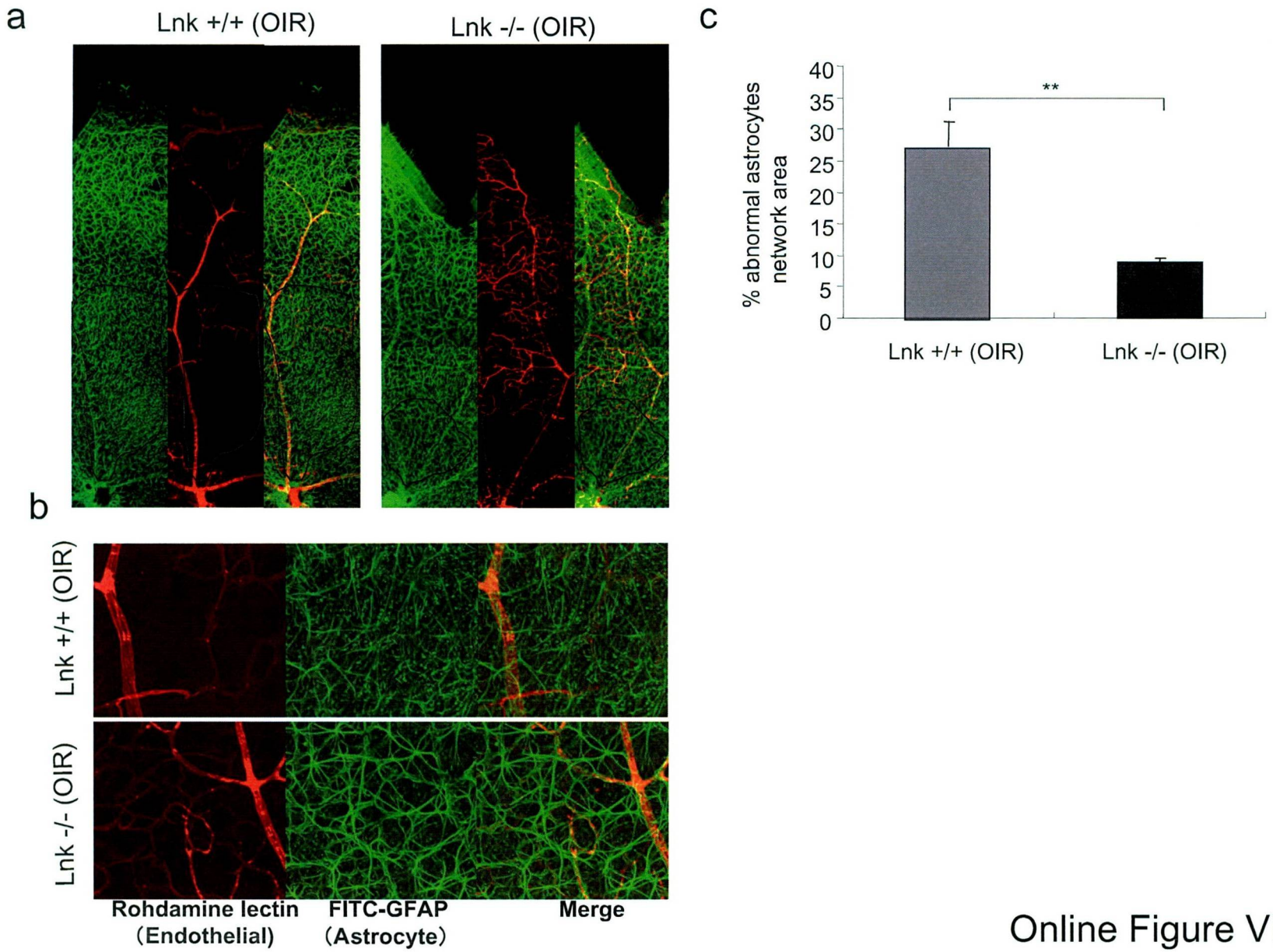

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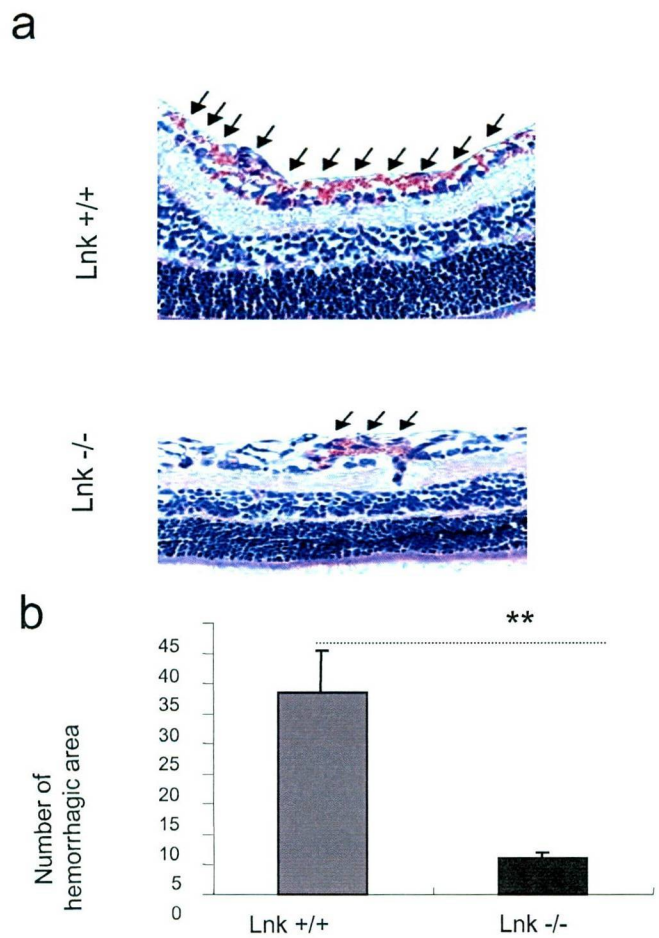

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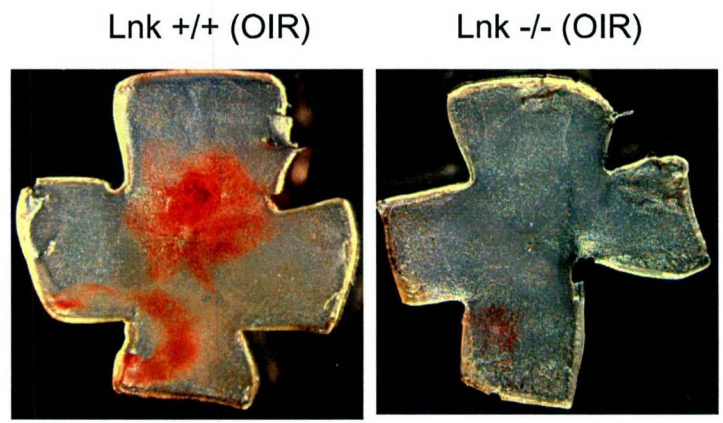

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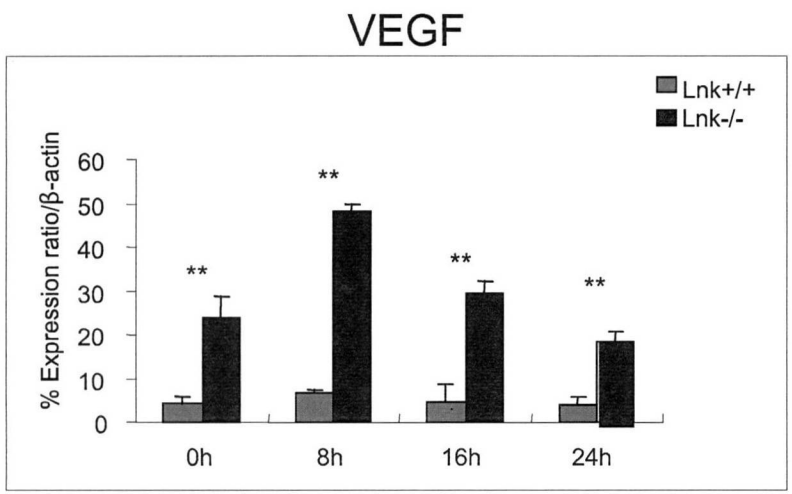

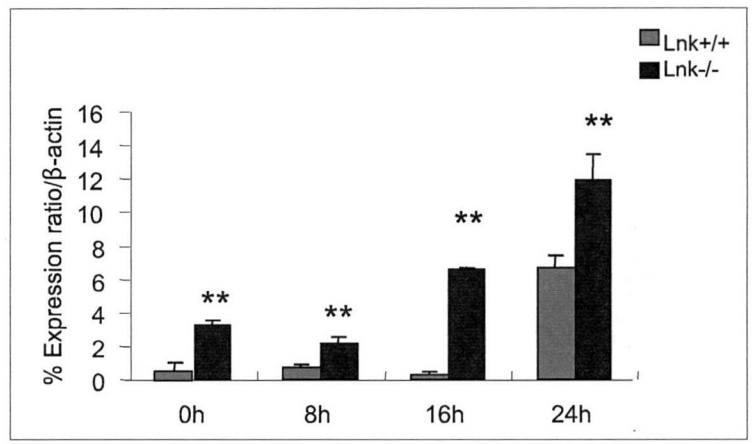

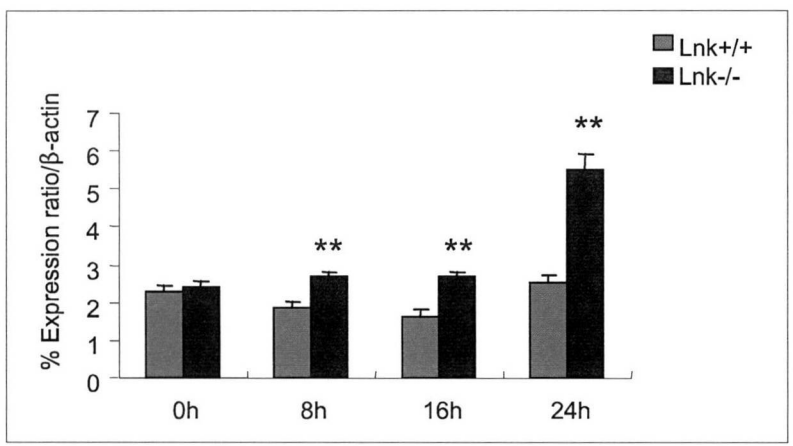

Online Figure X

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Concurrent Vasculogenesis and Neurogenesis From Adult Neural Stem Cells

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

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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.[1] 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,[2] as well as skeletal muscle cells,[3] and blood cells[4] in vitro. Similarly, after coculture with embryonic stem cells, NSCs differentiate into several lineage cells beyond germ lines when transplanted into an early-stage embryo.[5] The origin of endothelial cells, critical for both blood vessel formation that provides tissue sustenance and possibly organ induction and/or remodeling[6,7] 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,[8] 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.[9,10] The isolated NSCs were passaged suspending at a density of <2500 cells/cm^2 or <5 cells/mL.[8] 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.[5] 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.

Non-standard Abbreviations and Acronyms

Ang	angiopoietin
β-gal	β-galactosidase
BS	bandeiraea simplicifolia
DiI	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate
FITC	fluorescein isothiocyanate
HIF	hypoxia inducible factor
MAP	microtubule-associated protein
NSC	neural stem cell
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
NGF	nerve growth factor
NRP	neurophilin
SM	smooth muscle
VEGF	vascular endothelial growth factor

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)-1α 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).

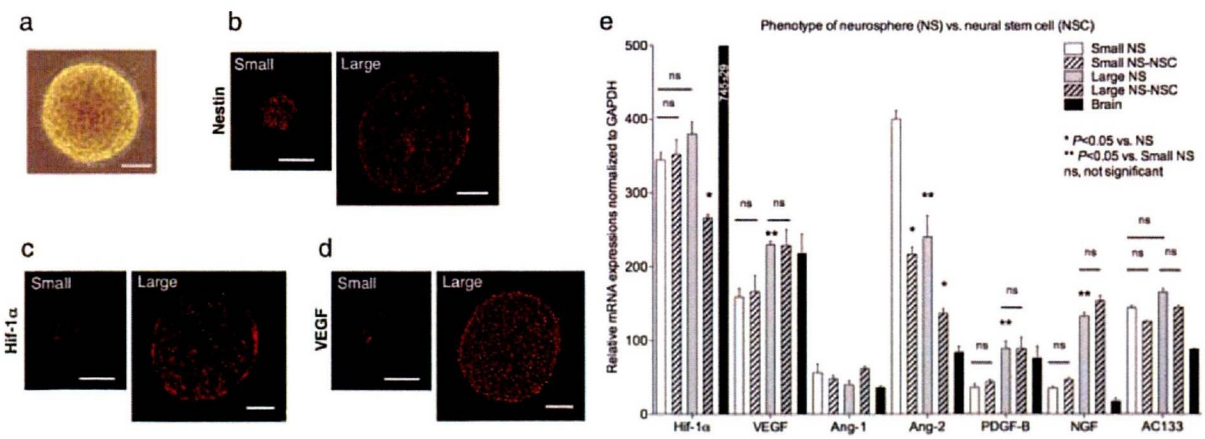

Figure 1. Characterization of neurospheres by the expressions of VEGF, Ang 2, PDGF-B, and AC133. a, Phase-contrast photomicrograph of a neurosphere on day 14 after isolation. Immunostaining of both small and large neurosphere for nestin (b), Hif-1a (c), and VEGF (d). Scale bars=100 μm. e, Gene expressions 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. Periventricular 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.

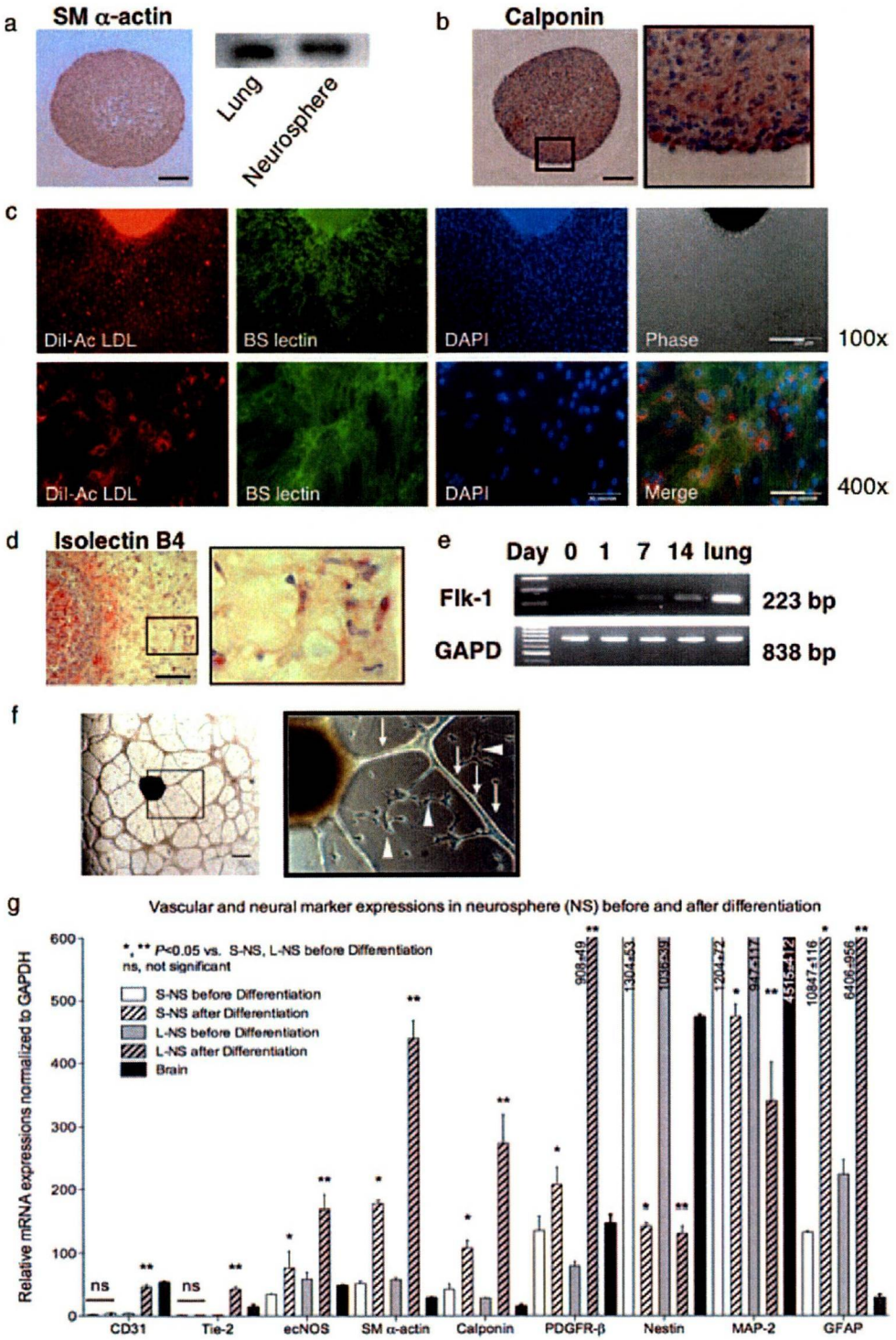

Figure 2. Analysis of vascular SM and endothelial lineage features of attached colony derived from neurospheres. a, Expression of SM α-actin in large neurospheres. Immunostaining (red) (left) and Northern analysis (right) documents that large neurospheres express SM α-actin. Scale bar: 100 μm. Total RNA from lung tissue was also loaded for positive control. b, Staining of large neurosphere with calponin (red). Scale bar: 100 μm. 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 μm for upper and lower images, respectively. d, Staining of attached large neurospheres with isolectin B4 (red). Boxed area is magnified. Scale bar: 200 μm. 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′,3′-tetramethylindo-carbocyanine perchlorate (DiI)-labeled acetylated low-density lipoprotein and were stained positively for fluorescein isothiocyanate (FITC)-labeled *Griffonia* (*bandeiraea*) simplicifolia lectin I (BS lectin) (Figure 2c). Large neurospheres cultured on Matrigel (BD) under same culture conditions were also shown to be positive for isolectin 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[21] 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)[22] was strikingly upregulated, whereas nestin (neural stem cell marker) and microtubule-associated protein (MAP)-2 (neuron-specific marker)[23] 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.[24] 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.[25] The clonally obtained secondary small neurospheres were cultured under the condition for neural differentiation[14] 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,[25,26] as well as certain particular neural cell types, such as dopaminergic neurons in midbrain[27] and astrocytes in subventricular zone.[28] These genes were clearly detected by RT-PCR analysis (Figure 3, lane 3). Furthermore, VEGF upregulated endothelial-relating ligands, Jagged-2[26] 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 neurophilin (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,[29] NGF signaling might be indirectly activated via sema-

Figure 2 (Continued). with endothelial differentiating medium for 2 weeks on rat vitronectin coating. A net-like structure (arrows) harbored neuron-like cells (arrowheads). Boxed area is magnified at the right. Scale bar: 200 μm. g, Gene expressions before and after differentiation of neurospheres into vascular and neural lineages. Small (S) and large (L) neurospheres were cultured on Matrigel with vascular cell–oriented medium for 7 days, and RNA samples were examined by real-time RT-PCR. 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. Small and large neurospheres were manually selected under microscopic observation.

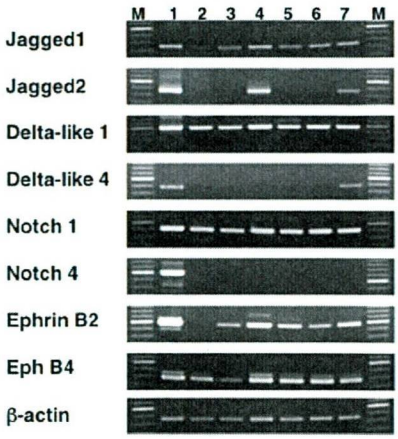

Figure 3. Analysis of vascular and neural cell–related ligands/receptors. a, RT-PCR analysis of ligands of notch and ephrin system. Total RNA was extracted from periventricular area of forebrain (lane 1), from unstimulated primary neurospheres with radii of <100 μm (lane 2), and from primary neurospheres cultured with 1% FBS-NSC medium without growth factor on PLO/laminin dish (lane 3) for 8 hours. The indicated materials were added to the medium (lanes 3 to 7). Dense band of the size marker corresponds to 600 base pairs (lane M). cDNA for β-actin was amplified in parallel reactions to assess cDNA-loading equivalence among samples. NGF-neutralizing antibody upregulated the expressions of endothelial-related ligands jagged-2 and Delta-like 4 (lane 7).

phorin 3A/NRP-1 binding. (3) Because NGF upregulates VEGF and its receptor expressions including NRP-1 in certain cell types,[30,31] 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)[25] 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 administered 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 (10^5/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-

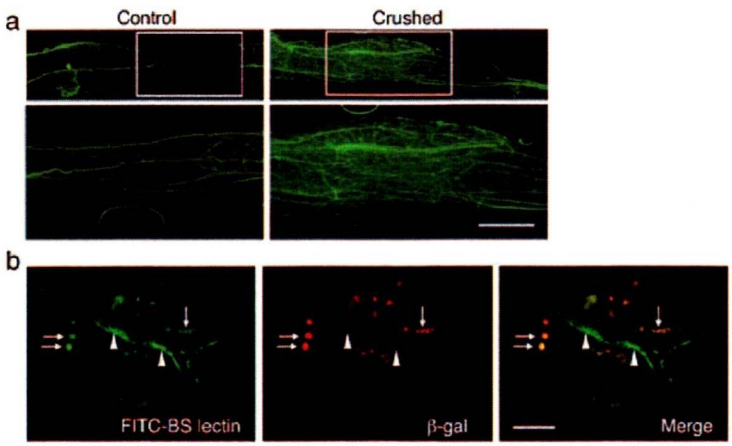

Figure 4. Whole mount immunostaining of crushed sciatic nerves. The sciatic nerve of 1 hindlimb was crushed at midthigh level; the contralateral limb side was used for a sham procedure. The dissociated NSCs from Rosa 26 mice were then administered (1×10^5 cells/mouse). After 2 weeks, the sciatic nerves were examined. a, Induction of neoangiogenesis by crush. Vasculatures were visualized by systemically perfused FITC-conjugated BS lectin, which binds to murine endothelial cells specifically, just before euthanasia. Boxed area is magnified. Scale bar: 200 μm. b, Identification of vasculature and cells derived from transplanted NSCs by immunostaining for BS lectin (green) and β-gal (red), respectively. Arrows indicate double-positive portion of the vasculature. Arrowheads denote BS lectin–positive but β-gal–negative portions. Scale bar: 50 μm.