Pivotal Role of Lnk Adaptor Protein in Endothelial Progenitor Cell Biology for Vascular Regeneration

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Abstract—Despite the fact that endothelial progenitor cells (EPCs) are important for postnatal neovascularization, their origins, differentiation, and modulators are not clear. Here, we demonstrate that Lnk, a negative regulator of hematopoietic stem cell proliferation, controls endothelial commitment of c-Kit+/Sca-1-/Lineage- (KSL) subpopulations of bone marrow cells. The results of EPC colony-forming assays reveal that small (primitive) EPC colony formation by CD34- KSLs and large (definitive) EPC colony formation by CD34(dim) KSLs are more robust in Lnk-/- mice. In hindlimb ischemia, perfusion recovery is augmented in Lnk-/- mice through enhanced proliferation and mobilization of EPCs via c-Kit/stem cell factor. We found that Lnk-deficient EPCs are more potent actors than resident cells in hindlimb perfusion recovery and ischemic neovascularization, mainly via the activity of bone marrow-EPCs. Similarly, Lnk-/- mice show augmented retinal neovascularization and astrocyte network maturation without an increase in indicators of pathogenic angiogenesis in an in vivo model of retinopathy. Taken together, our results provide strong evidence that Lnk regulates bone marrow-EPC kinetics in vascular regeneration. Selective targeting of Lnk may be a safe and effective strategy to augment therapeutic neovascularization by EPC transplantation. (Circ Res. 2009;104:969-977.)

Key Words: endothelial progenitor cell • Lnk • vascular regeneration

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a more immature HSC subpopulation than KSL CD34\(^+\) cells.\(^9\) Importantly, earlier studies report that expression of \(lnk\) is strong in immature cells, ie, c-kit\(^+\)/Lin\(^-\) cells, as compared with relatively mature cells, ie, c-Kit\(^-\)/Lin\(^-\) cells.\(^10\) Accordingly, and because mouse BM-KSLs are capable of differentiating into both hematopoietic and endothelial lineage cells and contribute to postnatal vasculogenesis,\(^11-13\) Lnk may regulate the functional kinetics of EPCs. Lnk has also been suggested to act as a negative regulator of the stem cell factor (SCF)–c-Kit signaling pathway.\(^16\) SCF reportedly stimulates proliferation and differentiation of HSCs and mobilizes stem cell populations from BM into peripheral blood (PB) by binding with its receptor, c-Kit. The SCF–c-Kit signaling pathway also supports stem cell survival and motility.\(^14\) Moreover, EPCs are recruited via interaction with membrane-bound c-Kit, which is highly expressed on ECs in ischemic tissue.\(^15\) The c-Kit–positive cells recruited to ischemic tissue reconstitute the injured heart and vasculature, via to their ability to regulate the myocardial balance of angiogenic cytokines.\(^16\)

Here, we sought to test the hypothesis that a lack of Lnk signaling may enhance postnatal neovascularization via specific control of the SCF–c-Kit–mediated regenerative potential of EPCs. We provide in vitro and in vivo evidence that Lnk plays a pivotal role in specific modulation of EPCs in terms of cell growth, commitment into endothelial lineage cell types, mobilization from BM into PB, and recruitment to ischemic sites for neovascularization.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Mice**

The \(lnk^{-/-}\) mice were generated as previously reported.\(^8\) All animal care and experiments were conducted in accordance with the institutional guidelines of Tokai University School of Medicine, Isehara, Japan.

**EPC Kinetics**

EPC colony–forming assay (EPC-CFA), single cell–based EPC-CFA, mobilization of EPCs, and in vitro 5\(^\prime\) bromodeoxyuridine (BrdUrd) proliferation assay were performed.

**In Vivo Study**

**Hindlimb Ischemia Model and Cell Transplantation**

The mouse model of hindlimb ischemia was generated by ligating the proximal femoral artery of 8- to 10-week-old C57BL/6j or BalbC nude mice.

**BM Transplantation Model**

C57BL/6j mice were exposed to a lethal dose of total body irradiation (10 Gy) and inoculated intravenously with 1\(^\times\)10\(^6\) donor BM mononuclear cells (BM-MNCs).

**Murine Model of Oxygen-Induced Retinopathy**

Oxygen-induced retinopathy (OIR) was induced in C57BL/6j wild-type (WT) and \(lnk^{-/-}\) mice.

**Results**

**Deficiency of \(lnk\) Augments Endothelial Differentiation and Upregulates Cell Growth-Relating Signals in BM-KSL Subpopulations**

Although a previous report has clearly shown that self-renewal of BM-CD34\(^+\) KSLs for hematopoiesis is accelerated in \(lnk^{-/-}\) mice,\(^9\) the role of Lnk in ischemic vasculogenesis is unknown. We first examined \(lnk\) mRNA levels in various populations of BM cells and several organs of WT mice in the presence or absence of limb ischemia. Expression of \(lnk\) mRNA is strong in BM-CD34\(^+\) KSLs regardless of tissue ischemia. Expression of \(lnk\) is moderate in BM-CD34\(^+\) KSLs, a relatively differentiated population as compared with CD34\(^+\) KSLs. In contrast, \(lnk\) expression was faint in samples from BM-MNCs, BM-Lin\(^-\) cells, skeletal muscle, and spleen independently of ischemia. These results suggest that \(lnk\) is highly expressed in BM hematopoietic and endothelial progenitors but not in mature BM cells or other organs. The \(lnk\) expression levels were especially high in the immature fraction of BM-HSC/EPCs as compared to committing fractions (Figure 1a).

The pattern of expression of \(lnk\) suggests a role in differentiation of various subpopulations among BM-KSLs. To test this, we next compared the number of BM-KSLs and derivative subpopulations in \(lnk^{-/-}\) and WT mice. The number of KSLs, CD34\(^+\) KSLs, and CD34\(^{dim}\) KSLs, but not CD34\(^{high}\) KSLs, was significantly greater in \(lnk^{-/-}\) mice than in WT. These data suggest that deletion of \(lnk\) results in an increase in immature subpopulations of KSLs (Figure 1b). To compare the vasculogenic commitment of BM-KSLs in \(lnk^{-/-}\) mice versus WT, fluorescence-activated cell-sorting analysis for endothelial markers was performed. KSLs coexpressing Flk-1 or CXCR4 were more frequent in \(lnk^{-/-}\) mice than in WT (Figure 1c). Thus, loss of \(lnk\) appears to promote vasculogenic commitment, resulting in an increase in the EPC pool in BM. Similarly, the number of EPCs increased in PB of Lnk-deficient mice (Figures 1 and 2 in the online data supplement).

To further confirm the role of Lnk in differentiation of KSL subpopulations into endothelial lineage cells, we performed an EPC-CFA established recently in our laboratory. KSLs and their subpopulations can form 2 types of EPC colony clusters, small (primitive) and large (definitive) EPC colony clusters. Both cluster types are positive for uptake of acetyl LDL (Ac-LDL) and for expression of an EC-specific marker, isoelectin B4, as revealed by chemical staining. Additionally, both are positive for Flk-1 (VEGF receptor 2) and CD31 (platelet endothelial cell adhesion molecule-1), as revealed by immunocytochemistry (Online Figure III, a through d). Moreover, colony-derived cells express the endothelial markers Flk-1 and CD31 at high levels, as detected by flow cytometric analysis. Cells from large EPC clusters, which comprise more committed EPCs with spindle-like morphology, more frequently show Ac-LDL uptake and higher expression of Flk-1 and CD31 than cells from small EPC clusters (Online Figure III, c and d).

EPC-CFA was performed for each KSL subpopulation obtained from \(lnk^{-/-}\) or WT mice. The number of small EPC colonies derived from CD34\(^+\) KSLs was significantly greater in \(lnk^{-/-}\) mice than in WT, whereas the number derived from CD34\(^{dim}\) or CD34\(^{high}\) KSLs was similar in \(lnk^{-/-}\) and WT. In contrast, the number of large EPC colonies from CD34\(^+\) KSLs was similar in both groups, whereas the number from CD34\(^{dim}\) or CD34\(^{high}\) KSLs was significantly higher in \(lnk^{-/-}\) mice than WT (Figure 1d). These data suggest that Lnk deficiency increases the capacity of immature stem cells...
to form primitive EPCs and in the capacity of relatively mature progenitor cells to differentiate into definitive EPCs.

To compare cell growth of CD34^-/CD34(dim) KSLs from Lnk^-/- versus WT mice, we next analyzed SCF-dependent glycojen synthase kinase (GSK3β) phosphorylation, which is part of a signaling cascade indispensable for cell growth.17 The level of phosphorylation of GSK3β in CD34^-/CD34(dim) KSLs was enhanced and prolonged in the Lnk^-/- background relative to WT. This points to an important role for Lnk in the ability of immature HSC/EPCs to cell growth, as apparently controlled by upregulation of the SCF-dependent GSK3β signaling pathway (Figure 1e).

**Lnk Deficiency Upregulates Proliferation and Endothelial Commitment of EPCs Derived From KSL Populations in Culture**

To explore the function of Lnk in EPC biology in terms of cell proliferation and commitment, we isolated and cultured Lin^- cells, KSLs, and KSL subpopulations from WT and Lnk-deficient mice in a defined EPC culture system. In both Lnk^-/- and WT genetic backgrounds, KSLs in general, and CD34^- KSLs and CD34(dim) KSLs in particular, proliferated efficiently in culture for 1 week, whereas BM-Lin^- cells, and KSL subpopulations of WT mice preischemia and 3 days after hindlimb ischemia. In both KSL subpopulations by flow cytometric analysis in Lnk^-/- mice and WT mice (n=6), FACS analysis of BM-KSL cells from Lnk^-/- and WT mice. d, EPC-CFA to evaluate vascular regeneration capacity of BM-KSL subpopulations in Lnk-deficient and WT mice. Colony number was counted 10 to 12 days after incubation of 500 cells per dish (n=4). e, Phosphorylation of GSK3β on stimulation of 100 ng/mL SCF in CD34(high)/KSLs of Lnk^-/- and WT mice. *P<0.05, **P<0.01, ***P<0.001.

We next looked at cultured KSL subpopulations in Lnk^-/- and WT genetic backgrounds. The results of flow cytometric analysis reveal that cultured cells derived from CD34^- KSL or CD34(dim) KSL subpopulations in Lnk^-/- mice were more frequently positive for the endothelial lineage markers Flk-1/Sca-1 and CXCR4/Sca-1 than those from WT. However, the number of cells positive for the endothelial markers among cells cultured from the CD34(high) KSL subpopulation was similar for Lnk^-/- and WT (Online Figure IV, b and c).

To determine whether EPC development from KSL subpopulations occurs at the single-cell level, we sorted single cells from each subpopulation, cultured the cells ex vivo for 1 week, and then assayed the cells using EPC-CFA and flow cytometry. EPC-CFA revealed that the number of large EPC colonies derived from single CD34^- KSL or CD34(dim) KSL, but not a single CD34(high) KSL, was significantly greater when cells were derived from Lnk^-/- mice. In contrast, the number of small EPC colonies derived from single cells of all subpopulations was similar in the 2 groups (Online Figure IV, d). Flow cytometry also revealed that the frequency of Sca-1^+ /Flk-1^+ cells, an EPC-enriched population, among cultured cells derived from single CD34^- KSLs or CD34(dim) KSLs, but not single CD34(high) KSLs, was significantly higher in Lnk^-/- than WT (Online Figure IV, e).

**Lnk Deficiency Promotes Neovascularization In Vivo**

The in vitro data above suggest that negative modulation of Lnk gene expression may promote neovascularization in ischemic tissue. To test the in vivo effect of Lnk deficiency,
angiogenic factors and their receptors in KSL subpopulations.

To assess the mechanism of enhanced blood flow recovery in \(\text{lnk}^{-/-}\) mice, we first compared the mitotic capacity of EPC-enriched populations in the presence or absence of hindlimb ischemia in \(\text{lnk}^{-/-}\) and WT genetic backgrounds. The percentage of BM Sca-1\(^+\)/BrdUrd\(^-\) cells in Lin\(^-\) cells without ischemia tended to be greater in \(\text{lnk}^{-/-}\) mice than in WT, but the difference was not statistically significant. In contrast, the percentage of cycling EPCs 7 days after ischemia was significantly greater in \(\text{lnk}^{-/-}\) mice than in WT (Figure 2a and 2b).

As for the kinetics of PB-EPCs, the number of Sca-1\(^+\) MNCs, an EPC-enriched fraction, on days 3 and 7 after hindlimb ischemia was significantly increased in \(\text{lnk}^{-/-}\) mice as compared to WT (Figure 2c).

To clarify the potential of EPCs in \(\text{lnk}^{-/-}\) mice for ischemic neovascularization, we used RT-PCR to compare mRNA expression of angiogenic factors and their receptors in KSL subpopulations in the presence or absence of hindlimb ischemia in \(\text{lnk}^{-/-}\) and WT. In \(\text{lnk}^{-/-}\) mice, genes that encode angiogenic factors or their receptors, such as \text{vegf}, \text{ang-1}, \text{tie-1}, and \text{tie-2}, were highly expressed independently of ischemic condition, whereas \text{ang-2}, an antagonist of TIE-2 signaling, was constitutively downregulated. In contrast, most angiogenic genes, which are weakly expressed at baseline, were upregulated postischemia in WT (Online Figure V). These data suggest that Lnk regulates the production of angiogenic factors, which in turn enhances EPC proliferation, differentiation, migration, and mobilization.

As for the kinetics of PB-EPCs, the number of Sca-1\(^+\) MNCs, an EPC-enriched fraction, on days 3 and 7 after hindlimb ischemia was significantly increased in \(\text{lnk}^{-/-}\) mice as compared to WT (Figure 2c).

A caveat to the above is that enhanced neovascularization in \(\text{lnk}^{-/-}\) mice could be attributable to upregulation of angiogenic effects of resident cells as well as augmentation of BM-derived EPC kinetics. To clarify the proportional contribution of these mechanisms, we performed BM transplantation (BMT) with cells from \(\text{lnk}^{-/-}\) or WT, with donor cells marked with green fluorescent protein (GFP) transplanted...
into unmarked recipients. Perfusion in limb tissue at day 14 postischemia dramatically improved in WT mice that received Lnk-deficient BM cells (BMCs) as compared with WT mice receiving WT BMCs. Moreover, perfusion recovery in the hindlimb was significantly inhibited in \( lnk^{-/-} \) mice receiving WT BMCs as compared with \( lnk^{-/-} \) mice receiving Lnk-deficient BMCs. Importantly, hindlimb perfusion was similar in WT and \( lnk^{-/-} \) mice receiving WT BMCs. Similarly, perfusion recovery was not significantly different between WT and \( lnk^{-/-} \) mice receiving Lnk-deficient BMCs (Figure 3a).

Next, we detected BM-derived endothelial lineage cells incorporating into the ischemic region via immunohistochemical detection of GFP and CD31 or isolectin B4 (Figure 3b). The number of GFP⁺/CD31⁺ ECs in ischemic tissue was significantly greater in WT mice receiving Lnk-null BMCs than in those receiving WT BMCs. In addition, BM-derived ECs were more frequently observed in \( lnk^{-/-} \) mice receiving Lnk-deficient BMCs than in those receiving WT BMCs. Similar to what was observed in the hindlimb perfusion analysis, the number of BM-derived ECs was equivalent in WT mice receiving Lnk-deficient BMCs and \( lnk^{-/-} \) mice receiving Lnk-deficient BMCs, as well as in WT mice receiving WT BMCs and \( lnk^{-/-} \) mice receiving WT BMCs (Figure 3c). These results suggest that BM-derived EPCs are indispensable for enhanced neovascularization in \( lnk^{-/-} \) mice, whereas \( lnk \) deficiency in resident cells does not significantly contribute to ischemic neovascularization.

**Lnk Deficiency Enhances EPC Kinetics in Response to Ischemia-Related Cytokines**

To identify specific cytokines responsible for enhanced mobilization of BM-ECs in \( lnk^{-/-} \) mice, we investigated the effect of several potent bioactive factors on EPC mobilization in \( lnk^{-/-} \) and WT genetic backgrounds. To do this, we administered G-CSF, SDF-1α, SCF, VEGF, or PBS to mice once daily over 5 days and determined the number of PB-MNCs on day 7. In both \( lnk^{-/-} \) and WT mice, each factor resulted in a significant increase in the number of PB-MNCs as compared with mock treatment (PBS). The number of PB-MNCs after administration of each factor was significantly greater in \( lnk^{-/-} \) mice than in WT. Notably, SCF and VEGF led to a more than 4-fold difference in PB-MNC number in \( lnk^{-/-} \) versus WT (Figure 4a). The results of an EPC culture assay using PB-MNCs also revealed that the number of circulating EPCs detected after infusion of any of the factors tested significantly increased in \( lnk^{-/-} \) mice as compared with WT. This difference between the 2 groups was particularly remarkable following infusion of SCF or VEGF (Figure 4b). To evaluate the scale of the Lnk-dependent SCF effect on EPC mobilization, we next looked at cell kinetics over time after SCF infusion in \( lnk^{-/-} \) or WT mice. The results of serial quantification of PB-MNCs revealed a significant increase in PB-MNCs in \( lnk^{-/-} \) mice that was detectable at day 2 and reached a peak on day 6 (Figure 4c). Furthermore, the results of serial FACS analysis revealed a significant increase in the PB-EC–enriched cell fraction (ie, in Sca-1⁺/CD31⁺ or Sca-1⁻/VE-cadherin⁺ cells) that was detectable at day 0 and still observable at day 8 after initiation of SCF infusion in \( lnk^{-/-} \) mice, as compared with levels in WT (Figure 4d and 4e). We next performed an in vitro proliferation assay to ask whether SCF upregulates proliferative activity of EPCs in \( lnk^{-/-} \) mice as well as mobilization. In WT mice, SCF did not affect the mitotic activity of Sca-1⁺/Lin⁻ cells. In contrast, treatment with 10 ng/mL of SCF significantly augments proliferation of EPC-enriched fraction cells in \( lnk^{-/-} \) mice (Figure 4f). Taken together, these data suggest that ischemia-related cytokines, in particular SCF/c-kit, are critical for both proliferation and mobilization of EPCs in \( lnk^{-/-} \) mice.

**Lnk-Deficient EPCs Rescue Hindlimb Ischemia Following Therapeutic Administration**

To evaluate the therapeutic potential of \( lnk \) gene–modified EPCs in ischemic neovascularization, we isolated and intravenously transplanted BM Sca-1⁻/Lin⁻ cells from \( lnk^{-/-} \) or WT mice into nude mice with hindlimb ischemia. As shown...
in Figure 5a and 5b, transplantation of Lnk-null EPCs resulted in robust hindlimb perfusion as compared with WT-EPCs at equal dosing. The results of immunohistochemical analysis using the EC markers isolectin B4 and CD31 surface antigen clearly show that the capillary density at ischemic tissues is higher in animals receiving Lnk-deficient EPCs than in those receiving WT-EPCs or a mock treatment (PBS) control (Figure 5c and 5d and Online Figure VI).

**Lnk Deficiency Enhances Neonatal Revascularization in OIR**

We next sought to test the effect of Lnk on vascular regeneration in retinal vascular disease. To do this, we generated an animal model of neonatal retinopathy, OIR, by exposing $\text{lnk}^{-/-}$ or WT mice to 75% oxygen from postnatal day 7 (P7) to P12 (Figure 6a). In WT mice with OIR, avascular regions of the retina were readily apparent at P17. In contrast, $\text{lnk}^{-/-}$ mice with OIR had 4-fold smaller retinal avascular areas than WT (Figure 6b and 6c). We also observed functional regeneration of the astrocyte network, accompanied by upregulation of blood vessel regeneration, in $\text{lnk}^{-/-}$ mice (Online Figure VII), suggesting that enhanced neovascularization may contribute to preservation of retinal interstitial structure in the Lnk-deficient microenvironment.

Previous results suggest that enhanced angiogenesis/vasculogenesis in the retina may result in pathogenic side effects such as excess inflammation and abnormal blood vessel formation, eventually leading to retinal bleeding. However, 

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**Figure 4.** Effects of ischemia/angiogenesis-related cytokines on EPC kinetics are more robust in $\text{lnk}^{-/-}$ mice. a, Number of PB-MNCs at day 6 after initiation of VEGF, SDF-1, GCSF, SCF, or PBS in $\text{lnk}^{-/-}$ mice and WT (n=5). b, In vitro EPC culture assay at day 6 after initiation of VEGF, SDF-1, GCSF, SCF, or PBS in $\text{lnk}^{-/-}$ mice and WT (n=4). The numbers of EPCs capable of Ac-LDL uptake and positive for isolectin B4 were significantly higher in $\text{lnk}^{-/-}$ mice than WT. c and d, Time course of SCF-dependent mobilization kinetics in $\text{lnk}^{-/-}$ and WT mice (n=4 at each time point in each group). Number of circulating MNCs (c) and PB Sca-1+/CD31+ cells (d) was serially evaluated in both groups.

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**Figure 5.** Lnk-deficient EPCs potently induce therapeutic neovascularization. a, Representative LDPI imaging in nude mice with hindlimb ischemia receiving BM Sca-1+/Lin- cells from $\text{lnk}^{-/-}$ or WT mice. Arrows indicate recovery of hindlimb perfusion following Lnk-deficient EPC infusion. b, Time course of hindlimb perfusion recovery in nude mice receiving mock treatment (PBS), WT-EPCs, or $\text{lnk}^{-/-}$ EPCs (n=7). c, Representative capillary structure revealed by chemical staining for isolectin B4 in nude mice receiving mock treatment, WT-EPCs, or $\text{lnk}^{-/-}$ EPCs. d, Capillary density at day 14 after hindlimb ischemia (n=7).
histological examination of our treated OIR model tissue revealed a smaller number of abnormally sprouting vessels in \( \text{lnk}^{-/-} \) mice than in WT (Figure 6d through 6f). Moreover, the incidence of retinal hemorrhage at P17 was markedly lower in \( \text{lnk}^{-/-} \) mice than in WT (Online Figures VIII and IX). These results suggest that \( \text{lnk} \) deficiency leads to an accelerated rate of retinal neovascularization without stimulating pathogenic blood vessel formation. To investigate this further, we isolated tissue from \( \text{lnk} \) deficient mice with OIR and used laser microdissection to look at the production of angiogenic growth factors in situ. Levels of VEGF, angiopoietin-1, eNOS, and leukemia inhibitory factor in vascular plexuses were significantly higher in \( \text{lnk}^{-/-} \) mice than in WT (Figure 7b). Importantly, enhanced expression of \( \text{ang}-1 \) may inhibit pathogenic angiogenesis by inducing the maturation of newly formed blood vessels.20 The source of angiogenic cytokines in Lnk-null OIR is likely to be at least in part BM-derived EPCs that are recruited into the retina, as both EPCs cultured in vitro under hypoxic conditions (Online Figure X).

**Discussion**

The results of previous studies12,21,22 have clearly demonstrated that BM-derived hematopoietic stem cells such as BM-KSLs serve as a reservoir of EPC origin cells in adults. In addition to having a long-term capacity for multilineage hematopoiesis, transplanted KSLs have also been shown to give rise to functional endothelial cells, even after single-cell transplantation or serial transplantation in the presence of retinal ischemic injury.12,21 Although differentiation of hematopoietic and endothelial lineages has been intensively inves-
expression of Lnk in stem cells and the capacity of Lnk to control lineage commitment/differentiation of BM stem cells suggest a pivotal role for Lnk as a regulator of EPCs in adults.

In addition to suggesting roles for Lnk in EPC commitment and differentiation, the results presented here also indicate that Lnk deficiency results in higher levels of proliferation of BM-KSLs and their subpopulations in vitro. Thus, we used an animal model of hindlimb ischemia to assess the effects of Lnk deficiency on EPC kinetics in vivo. Lnk deficiency results in enhanced recovery of hindlimb perfusion via upregulated proliferation of BM-derived EPCs, their enhanced mobilization activity into PB, and markedly increased recruitment into sites of ischemia. These data strongly suggest that both production of quiescent stem cells in the BM and the supply of stem cells from the BM pool for ischemic vasculogenesis may be controlled by Lnk. Furthermore, overexpression of angiogenic cytokines in Lnk-deficient KSL subpopulations suggests the importance of paracrine effects of KSL subpopulations for in situ angiogenesis as well as their autocrine effect for direct vasculogenesis. Interestingly, the results of a series of BMT experiments show that Lnk deficiency in BM-derived EPCs, but not resident EPCs/ECs, specifically augments neovascularization post hindlimb ischemia. These results provide the first direct evidence that the Lnk adapter protein plays a pivotal role in regulating the bioactivities of BM-derived EPCs for postnatal neovascularization.

Using OIR as a model for retinal damage, we also found that signs of pathogenic angiogenesis in the retina, such as tuft formation and retinal hemorrhage, were much lower in Lnk-deficient mice than in WT. Regeneration of a mature astrocyte network, along with robust neovascularization in Lnk<sup>−/−</sup> mice, further supports the idea that knockdown of Lnk can have a beneficial and nonpathogenic effect in retinal vascular disease (Figure 6a through 6h and Online Figures VIII and IX). This notion may be explained by the beneficial effects of Ang-1 stimulation of vessel maturation. Consistent with this, quantitative RT-PCR using microdissected retinal tissue revealed higher levels of expression of ang-1 and other angiogenic cytokines, VEGF and eNOS, in Lnk-null mice than in WT (Figure 7b).

In conclusion, we provide strong evidence that Lnk is a definitive regulator of BM-EPC kinetics, including the ability to cell growth, endothelial commitment, mobilization, and recruitment for vascular regeneration. Selective targeting of Lnk may be a safe and effective approach to augment therapeutic neovascularization by EPC transplantation.

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**Disclosures**

None.

**References**


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ONLINE MATERIALS AND METHODS

Mice. The \( \text{Ink}^+ \) mice were generated as previously reported \(^1\). We purchased C57BL/6J from Clea Japan Inc. For the generation of \( \text{Ink}^+ \) mice expressing GFP, the \( \text{Ink}^+ \) mice and GFP transgenic mice were bled and genotyped as previously reported \(^1\). All mice were maintained with the support of the animal care facility in accordance with the regulations of Tokai University and RIKEN Center for Developmental Biology.

**EPC colony forming assay (EPC-CFA).** KSLs and their CD34 subpopulations (CD34\(^{+}\), CD34\(^{\text{dim}}\) and CD34\(^{\text{high}}\)) were isolated from BM using FACS Vantage sorting equipment (Becton Dickinson) at more than 98% of purity. PB-MNCs were isolated by density-gradient centrifugation with Histopaque 1083 (Sigma) as previously described \(^2\ \!\!^3\ \!\!^4\ \!\!^5\). The frequency of small or large type EPC colony formation was assessed in methylcellulose-containing medium M3236 (StemCell Technologies) with 20 ng/ml SCF (Kirin), 50 ng/ml VEGF (R&D Systems, Minneapolis, Minn), 20 ng/ml interleukin-3 (Kirin), 50 ng/ml basic fibroblast growth factor (bFGF, Wako), 50 ng/ml epidermal growth factor (EGF, Wako), 50 ng/ml insulin-like growth factor-1 (IGF, Wako) and 2 U/ml heparin (Ajinomoto).

**Expansion of EPC-enriched cells.** Ex vivo expansion of EPC-enriched cells was performed according to the procedures established in our laboratory (Masuda et al, not published). In brief, 1 x 10^4 freshly isolated cells were cultured in Stem Span media (StemCell Technologies) without serum, supplemented with 50 ng/ml VEGF (R&D Systems), 20 ng/ml interleukin-6 (R&D Systems), 100 ng/ml SCF (Kirin), 20 ng/ml thrombopoietin (TPO) (Wako), 100 ng/ml Flt-3 ligand (Wako) and antibiotics. After 7 days in culture, the cells were removed by washing with PBS and used for flow cytometry analysis, EPC-CFA or proliferation assay.

**Single cell-based EPC-CFA.** Freshly isolated single cell from CD34\(^{+}\) KSL cells in each well of 96-well plates was expanded into committed EPCs (more than 100 cells) for 1 week similarly as in the EPC expansion described above. Expanded cells were recultured into media of EPC-CFA in 24 well plates with 300 \( \mu \)l of methylcellulose-containing medium (M3236) supplemented with various cytokines as described.
above. Small EPC-dominant or large EPC-dominant colonies were microscopically identified in every sample. By flow cytometry, the number of Sca-1(+) Flk-1(+) cells in the expanded EPCs was evaluated at day 7.

Flow cytometry. For flow cytometry analysis, we used monoclonal antibodies specific for the following surface antigens: Sca-1 (Becton Dickinson), c-Kit (Becton Dickinson), CD34 (Becton Dickinson), Flk-1 (Becton Dickinson), CXCR4 (e-bioscience), CD31 (Becton Dickinson), VE-cadherin (Becton Dickinson). We used a mixture of biotinylated monoclonal antibodies against B220 (RA3-6B2), CD3 (145-2C11), CD11b (M1/70), TER-119 (Ly-76), Gr-1 (RB6-8C5) from BD pharmingen as lineage markers to deplete lineage-positive cells from BM-derived cells. Propidium iodide (Becton Dickinson) was dissolved at 3 mM in PBS and used at 30 µM. The cells were analyzed by two-color flow cytometry using a FACS Caliber (Becton Dickinson).

Hindlimb ischemia model and cell transplantation. The mouse model of hindlimb ischemia was generated by ligating the proximal femoral artery of eight- to ten-week-old C57BL6/J or Balb/C nude mice. To prepare for BM-derived EPC-enriched fraction for transplantation, total BM cells were harvested aseptically by flushing tibias and femurs of Lnk-null or WT mice, and BM-MNCs were isolated by density gradient centrifugation. After staining with the lineage marker antibody mixture as described above, MACS and FACS sorting were performed to purify a Sca-1(+) Lin(-) population. Then, animals received 2.5 x 10^5 freshly-isolated BM Sca-1(+) Lin(-) cells, EPC-enriched cells, by an intravenous injection. To evaluate limb perfusion ratio (ischemic limb (right)/ normal limb (left)), a laser Doppler perfusion imaging (LDPI, Moor Instruments) was performed at days 4, 7, 14 and 28 post ischemia as previously described 6.

BM transplantation (BMT) model. C57BL/6J mice were exposed to a lethal dose of total body irradiation (10 Gy) and inoculated intravenously with 1 x 10^6 donor BM-MNCs obtained from Lnk-deficient mice (homozygous mice with GFP expression) or control mice expressing GFP. At 6 week post transplantation, FACS analysis revealed that frequency of GFP(+) cells in the BM-MNCs, an indicator of donor-cell chimerism, was >80% in all recipient animals.
In vitro EPC culture assay. PB-MNCs were isolated by density-gradient centrifugation using Histopaque 1083 (Sigma) 4 or 7 days after hindlimb ischemia and were characterized as previously described. Briefly, 1 x 10^5 PB-MNCs were incubated for 7 days in EBM2 medium, then 10 µg/ml of Dil-conjugated Ac-LDL (Biomedical Technologies Inc.) was added for 5 hours at 37°C. After washing, the cells were fixed and stained with FITC-conjugated isolectin B4, a chemical marker of ECs. The cells demonstrating both uptake of Ac-LDL and positivity for isolectin B4 were counted in four high-power fields randomly selected.

EPC kinetics. To evaluate cytokine-induced mobilization of BM cells into PB, mice were intraperitoneally injected with 10 µg/kg of SCF (Kirin), 2.5 µg/kg of VEGF (R&D Systems), 10 µg/kg of G-CSF (Kirin), 2.5 µg/ml of SDF-1 (R&D Systems), or a saline control once daily for 5 days. Number of PB-MNCs at days 0, 2, 4, 6 and 8 was scored. To characterize the EPC population, two separate experiments were performed. First, FACS analysis was performed using antibodies against Sca-1 (Becton Dickinson), CD31 (Becton Dickinson), and VE-cadherin (Becton Dickinson). Second, EPC culture assay to evaluate capacity of metabolic uptake of Dil-AcLDL and positivity for isolectin B4, was tested using fluorescence microscopy.

We also examined EPC kinetics after hindlimb ischemia. Number of PB EPC-enriched fraction was counted as described above. In addition, an in vitro EPC culture assay as well as an EPC-CFA, wherein 7 x 10^5 MNCs per samples were cultured for 10 days and scored by microscopic observation, was examined. To analyze EPC proliferation, we isolated the BM Lin(-) population in the presence or absence of hindlimb ischemia, then performed in vitro BrdU uptake analysis followed by staining for Sca-1 as previously described.

Murine model of oxygen-induced retinopathy (OIR). OIR was induced in C57/BL6 WT and lnk^-^- mice (C57/BL6 genetic background) as described by Smith and colleagues. In brief, neonatal mice were exposed to 75% oxygen in an incubator (BioSpherix, Redfield, NY) between postnatal day seven (P7) and P12, producing vaso-obliteration and cessation of vascular development in the capillary beds of the central retina. Animals were returned to room air at P12, by which time they had ischemia and hypoxia in the central retinal tissue. Maximal neovascularization was...
seen at P17. For whole mount preparations, retinas were isolated and fixed in 4% paraformaldehyde, followed by immunohistochemistry as previously described. For cryosections, eyes were frozen in OCT compound (Sakura Finetek) and sectioned at a thickness of 10 µm (flat-mount). Paraffin-embedded sections of the eyes (5 µm thickness) were stained with hematoxylin and eosin.

*Lectin labeling of retinal vasculature.* The retinal vasculature were imaged by perfusion-labeling with Rhodamine-conjugated concanavalin A lectin (Con A; Vector Laboratories). After deep anesthesia, the chest cavity was opened and a 27-gauge cannula was introduced into the left ventricle. After injection of 2 mL of PBS, 2 mL of Rhodamine-conjugated Con A lectin was perfused. After the eyes were enucleated, the retinas were flatmounted. The flatmounts were imaged by an epifluorescence microscope.

*Immunohistochemistry for whole mount retina.* For immunohistochemistry, whole mount retinas were fixed with 4% paraformaldehyde and blocked in 1% BSA / 0.3% TritonX / PBS for 1h at room temperature. Retinas were processed with primary antibodies as follows: anti-mouse PECAM-1 (BD Biosciences) at 1:500 dilution, anti-mouse GFAP (Dako) at 1:1000 dilution and anti-GFP (Molecular Probes) at 1:200 dilution. Secondary antibodies used were conjugated either to Alexa 488 or 594 (Molecular Probes) at 1:1000 dilution.

*Quantitative analysis of hemorrhagic area.* Flat-mount retinas fixed with 4% paraformaldehyde were photographed using a 10 x objective on microscope. To measure the area of hemorrhagic region and whole retina, a software, ImageJ (NIH, Bethesda, ML) was used. Ratio of hemorrhagic area with the whole retinal area was calculated.

*BM-derived EPC culture under hypoxic condition.* BM-MNCs isolated from tibia and femur were plated on cell culture dishes coated with rat vitronectin at a density of 5 x 10^5/cm² and cultured with 5% FBS/EBM-2 medium to obtain the EPC-enriched population. After 4 days, the putative EPCs were cultured under normoxic (19% O₂, 76% N₂ and 5% CO₂) or hypoxic (2% O₂, 93% N₂ and 5% CO₂) conditions for 8, 16, 24 hours in a standard incubator at 37°C.
Laser capture microdissection of vascular plexuses. Whole eyes were embedded in OCT compound (Sakura Finetek), snap-frozen in liquid nitrogen, and stored at -80°C. The frozen tissues were cut at 10 µm thickness and mounted on Leica foil slides (Leica microsystems). The frozen sections were fixed in ice-cold ethanol/acetic acid (19:1) for 3 min, rinsed in ice-cold DEPC water for 1 min, applied toluidine blue solution (0.05%) for 30 sec, rinsed twice in ice-cold DEPC water for 1 min, and air dried for 10 min. Laser capture was performed by removing the retinal vascular plexuses using AS LMD (Application Solutions Laser Microdissection System, Leica Microsystems).

Total RNA was extracted from the captured cells using the QUIAGEN RNeasy Micro Kit. cDNA synthesis from eluted RNA and amplification of cDNA were performed by Saito’s method\textsuperscript{10}. The amplified cDNAs with 60-fold dilution were used for quantitative RT-PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers in an Oligo software (Takara). The mean cycle threshold (Ct) values from quadruplicate measurements were used to calculate the gene expression with normalization to GAPDH as an internal control.

Primers:

\begin{itemize}
  \item mLIF: forward (5TGG GGC ATT TTG CAG TTT AT 3), reverse (5AGT CGG GAT CAA GGA CAC AG 3)
  \item mAng1: forward (5AAC CTC ACC CTG CAA AGA TG 3), reverse (5CAC AGA TGG CCT TGA TGT TG 3)
  \item meNOS: forward (5TAC GCA CCC AGA GCT TTT CT 3), reverse (5GAA CTG GAG GGG AGG AAG AC 3)
  \item mVEGF: forward (5TCT CCC AGA TCG GTG ACA GT 3), reverse (5GGG CAG AGC TGA GTG TTA GC 3)
  \item mGAPDH: forward (5TGT GTC CGT CGT GGA TCT GA 3), reverse (5ACC ACC TTC TTG ATG TCA TCA TAC TT 3)
  \item mb-actin: forward (5AAG TCC CTC ACC CTC CCA AAA G 3), reverse (5AAG CAA TGC TGT CAC CT TCC 3)
\end{itemize}

Incorporation of EPCs in retina. To evaluate the incorporation of EPCs in ischemic retina, P12 WT mice with OIR received an intraperitoneal transplantation of 5 x 10\textsuperscript{5} BM Sca-1\textsuperscript{(+)}/Lin\textsuperscript{(-)} cells obtained from Lnk-null or WT mice encoding GFP.
Histological density of the incorporated EPCs was quantified using the confocal images (× 20 objective lens) of the superficial central retina, including a quadrant of the optic nerve head as a point of reference.

**Western blotting.** Purified BM-derived Sca-1(+)/Lin(-) cells at day 0 or 7 after hindlimb ischemia were immediately lysed in lysis buffer and subjected to immunoblot analysis. The following antibodies were used: mouse Akt, mouse phospho-Akt, mouse eNOS, mouse phospho-eNOS (Cell Signaling Technology) and mouse actin (Sigma) antibodies.

**RT-PCR.** We obtained total RNAs from KSLs and from the CD34+/dim+/high KSL subpopulations of Lnk-deficient or WT mice before and after hindlimb ischemia, then produced first-strand cDNAs. To quantify the transcripts, semi-quantitative RT-PCR reactions were performed and normalized to Actb, which encodes beta actin. PCR reactions were performed at 94°C for 45 seconds, 60°C for 1 minute and 72°C for 1 minute for 35 or 25 cycles and analyzed on 2% agarose gels. Some PCR reactions were performed with a 45 second annealing step at 64°C instead of 60°C.

**Histological Assessment.** ECs were stained with rat-anti-mouse CD31 antibody (BD Biosciences) or isoelectin B4 (Vector Laboratory) using tissue sections from the lower calf muscles of ischemic limbs on day 28. Capillaries were recognized as tubular structure positive for CD31 or isoelectin B4. Capillary density was morphometrically evaluated by histological examination of 5 randomly selected fields of the tissue sections.

**Statistical analysis.** All values were expressed as mean ± SEM. The results were statistically analyzed with the use of a software package, Statview 5.0 (Abacus Concepts Inc, Berkeley, CA). Student's unpaired t test was performed for the comparison of items between Lnk-null mice and WT mice. The Scheffe's test was performed for the multiple comparisons after ANOVA between each group. P < 0.05 was considered statistically significant.
ONLINE FIGURE LEGENDS

Online Figure I: Flow cytometric analysis of PB-MNCs in $lnk^{-/-}$ and WT mice for endothelial markers. Frequency of Sca-1(+)/CD31(+) or Sca-1(+) or Sca-1(+) cells, which are EPC-enriched populations, was examined.

Online Figure II: In vivo EPC enrichment in BM and PB of $lnk^{-/-}$ mice. (a) Number of BM CD34(+), Flk-1(+) or CXCR4(+) KSLs (/ mouse) was compared between $lnk^{-/-}$ and WT mice (n=4 in each group). (b) Number of circulating CD31(+)/Flk-1(+)/VE-cadherin(+)/ or CXCR4(+)/Sca-1(+) cells (/ mouse), EPC-enriched fractions, was evaluated in $lnk^{-/-}$ mice or control mice. (n=4 in each group).

Online Figure III: Establishment of EPC colony forming assay (CFA) (a) Representative morphology of small and large EPC colonies. Small EPC colony consists of clusters of both small and round-shaped cells, indicating the colony of primitive EPCs (left panel), whereas large EPC colony demonstrates clusters of relatively large and spindle-shaped cells, suggesting the colony of definitive EPCs (right panel). (b) Both small and large EPC clusters were capable of uptaking Ac-LDL and strongly expressing endothelial surface marker, isolectin B4. (c,d) Small and large EPC colonies were further characterized by flow cytometry for DiI-conjugated Ac-LDL and FITC-conjugated isolectin B4 (c), and for other EPC markers, Flk-1 (VEGFR2) and CD31 (PECAM) (d).

Online Figure IV: Increased potential for proliferation and EPC commitment in BM KSL subpopulations of $lnk^{-/-}$ mice demonstrated by ex vivo expansion study. Freshly isolated CD34 KSL subpopulations (CD34(+/−), CD34(dim) and CD34(high)) were expanded in culture for 1 week (a). Proliferative capacity of the BM KSL subpopulations obtained from $lnk^{-/-}$ or WT mice during the ex vivo expansion (n=4). (b) Expanded BM KSL subpopulations for 7 days were then subjected to flow cytometry (c) (n=4). Single cell-based evaluation of endothelial differentiation capacity of BM KSL subpopulations in Lnk-null or WT mice. Single cell isolated from each KSL subpopulation was expanded in EPC expansion medium (see online MATERIALS and METHODS) for 1 week. Endothelial commitment of the expanded cells derived from each single cell was further determined by EPC CFA.
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).
SUPPLEMENTARY REFERENCES


BM–MNCs-derived EPC enriched Cells

KSL CD34(-)  KSL Flk-1(+)  KSL CXCR4(+)

Lnk +/+  Lnk -/-

KSL CD34(-)  KSL Flk-1(+)  KSL CXCR4(+)

CD31(+)  Flk-1(+)  VE-cad(+)  CXCR4(+)

PB-MNCs-derived EPC enriched Cells (in Sca-1 + cells)

Online Figure II
Online Figure III
Large EPC colony
Small EPC colony

VEGF, SCF, Flt3, TPO, Flt3 in SF culture condition

KSLCD34 (-) (dim) (high)
Lnk (+) (+) (+) (+) (+)

Flk-1 (VEGFR2)/ Sca-1
CXCR4/ Sca-1

Online Figure IV
Online Figure V

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Online Figure VI
Online Figure VII
Online Figure VIII

(a) Images showing hemorrhage areas in Lnк +/+ and Lnк -/- mice. Arrows indicate hemorrhage sites.

(b) Bar graph comparing the number of hemorrhagic areas in Lnк +/+ and Lnк -/- mice. ** indicates statistical significance.
Online Figure IX
**Online Figure X**

**VEGF**

- **Lnk+/+**
- **Lnk−/−**

**eNOS**

- **Lnk+/+**
- **Lnk−/−**

**Ang-1**

- **Lnk+/+**
- **Lnk−/−**

% Expression ratio/β-actin

- 0h
- 8h
- 16h
- 24h

- **0**
- **10**
- **20**
- **30**
- **40**
- **50**
- **60**

- **0**
- **2**
- **4**
- **6**
- **8**
- **10**
- **12**
- **14**
- **16**

**Online Figure X**