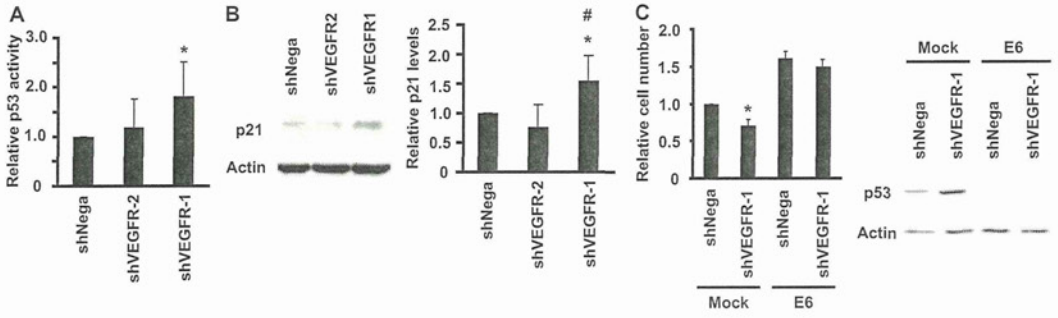

Figure 2. VEGFR-1 deletion induces activation of the p53/p21 signal pathway. A, A luciferase reporter gene plasmid (pPG13-Luc) containing the p53-binding sequence was transfected into endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Luciferase activity was measured at 48 hours after transfection in the presence of VEGF-A (10 ng/mL) as described in Methods. *$P<0.05$ vs shNega (n=5). B, Whole cell lysates (30 μg) were prepared from infected endothelial cells and p21 expression was assessed by Western blot analysis. *$P<0.05$ vs shNega, #$P<0.01$ vs shVEGFR-2 (n=4). C, Human endothelial cells were infected with pLNCX (Mock) or pLNCX E6 (E6). Infected cell populations were then transduced with shNega or shVEGFR-1. After purification, double-infected cells were seeded at a density of 2×10^5 cells per 100-mm dish in the presence of VEGF-A (day 0), and cell number was counted on day 3. *$P<0.05$ vs Mock/shNega (n=3). Western blot analysis revealed that introduction of E6 effectively ablated p53 expression (right panel).

Influence of VEGFR-1 Deletion on Neovascularization In Vivo

To examine the influence of VEGFR-1 deletion on neovascularization in vivo, we produced a hindlimb ischemia model in VEGFR-$1^{+/-}$ mice and assessed blood flow recovery and the capillary density of ischemic tissue. VEGFR-1 mRNA levels were significantly lower in VEGFR-$1^{+/-}$ mice than in wild-type mice (Figure 4A). Aortic expression of VEGFR-1 protein was decreased in VEGFR-$1^{+/-}$ mice compared with wild-type mice (Figure 4B). Consistent with the in vitro data, phospho-Akt levels were significantly higher in VEGFR-$1^{+/-}$ mice than in wild-type mice (Figure 4C and supplemental Figure III). There was no significant difference in plasma VEGF levels between the two groups (data not shown). Laser Doppler image analysis revealed that blood flow recovery was significantly impaired in VEGFR-$1^{+/-}$ mice compared with their wild-type littermates (Figure 4D). Likewise, VEGFR-$1^{+/-}$ mice exhibited significantly fewer CD31-positive cells in the ischemic tissues than their wild-type littermates (Figure 4E), suggesting that decreased expression of VEGFR-1 led to reduced neovascularization of ischemic tissue.

There are several reports indicating that VEGFR-1 kinase activity is required for VEGF-induced migration of hematopoietic cells including macrophages,[21–26] and it was reported that infiltration of macrophages plays a critical role in pathological angiogenesis during ischemia, inflammation, and tumor development.[27–29] Therefore, we examined the number of infiltrating macrophages in ischemic tissue, but we found no significant difference in the number of Mac3-

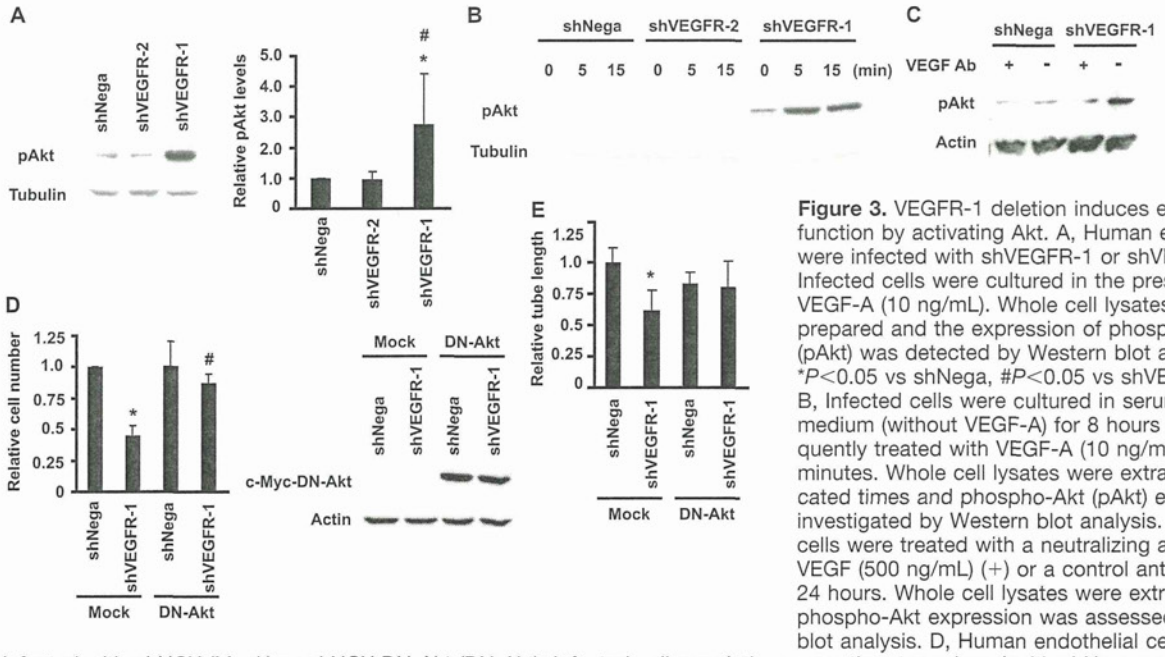

Figure 3. VEGFR-1 deletion induces endothelial dysfunction by activating Akt. A, Human endothelial cells were infected with shVEGFR-1 or shVEGFR-2. Infected cells were cultured in the presence of VEGF-A (10 ng/mL). Whole cell lysates (30 μg) were prepared and the expression of phosphorylated Akt (pAkt) was detected by Western blot analysis. *$P<0.05$ vs shNega, #$P<0.05$ vs shVEGFR-2 (n=5). B, Infected cells were cultured in serum-free basal medium (without VEGF-A) for 8 hours and subsequently treated with VEGF-A (10 ng/mL) for 5 to 15 minutes. Whole cell lysates were extracted at indicated times and phospho-Akt (pAkt) expression was investigated by Western blot analysis. C, Infected cells were treated with a neutralizing antibody for VEGF (500 ng/mL) (+) or a control antibody (−) for 24 hours. Whole cell lysates were extracted and phospho-Akt expression was assessed by Western blot analysis. D, Human endothelial cells were infected with pLNCX (Mock) or pLNCX DN-Akt (DN-Akt). Infected cell populations were then transduced with shNega or shVEGFR-1 and were subjected to the proliferation assay as described in legend for Figure 2C. *$P<0.005$ vs Mock/shNega, #$P<0.005$ vs Mock/shVEGFR-1 (n=6 to 8). Expression of c-Myc-tagged DN-Akt was confirmed by Western blot analysis (right panel). E, Double-infected endothelial cells (prepared as in Figure 3C) were subjected to the tube-forming assay. *$P<0.05$ vs Mock/shNega (n=3).

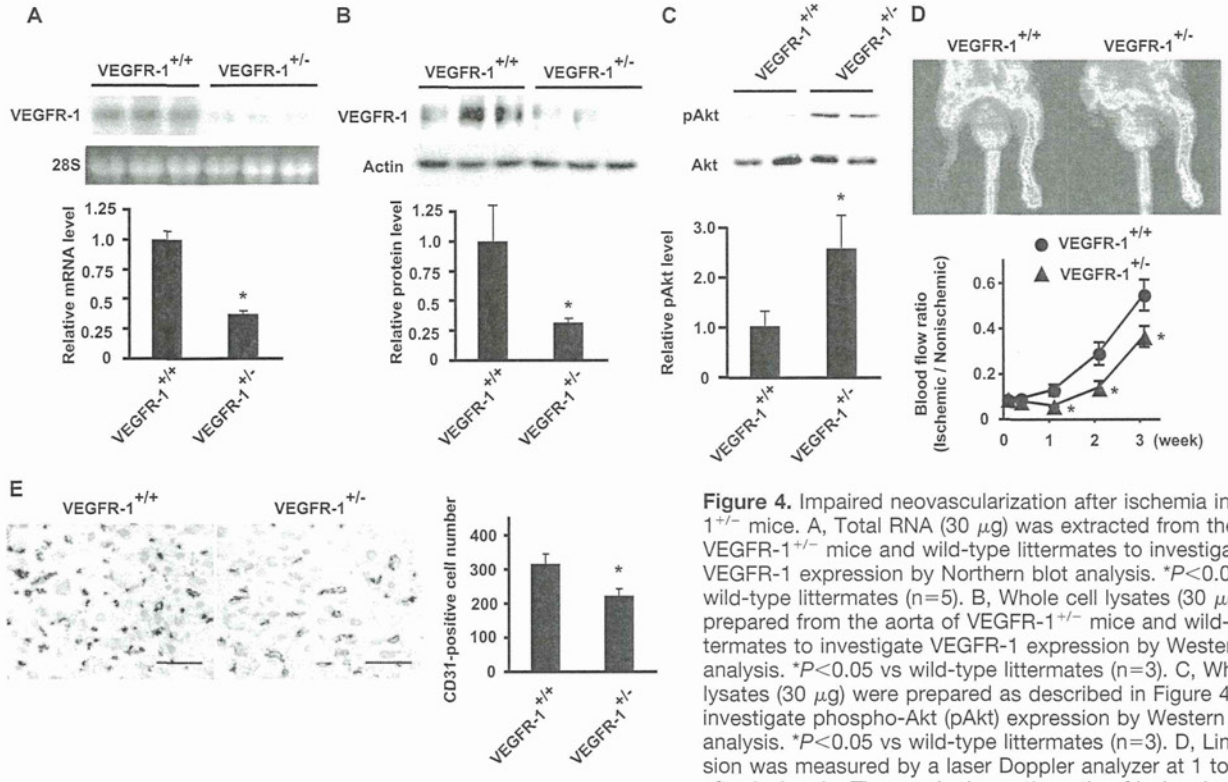

Figure 4. Impaired neovascularization after ischemia in VEGFR-$1^{+/-}$ mice. A, Total RNA (30 μg) was extracted from the lung of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Northern blot analysis. *$P<0.001$ vs wild-type littermates (n=5). B, Whole cell lysates (30 μg) were prepared from the aorta of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). C, Whole cell lysates (30 μg) were prepared as described in Figure 4B to investigate phospho-Akt (pAkt) expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). D, Limb perfusion was measured by a laser Doppler analyzer at 1 to 3 weeks after ischemia. The graph shows the ratio of ischemic (right) to nonischemic limb (left) blood flow. *$P<0.05$ vs wild-type littermates (n=16). E, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. The number of CD31-positive cells per square millimeter is shown in the graph. *$P<0.05$ vs wild-type littermates (n=4).

positive cells between VEGFR-$1^{+/-}$ mice and their wild-type littermates (Figure 5A). To further test the possible involvement of bone marrow–derived cells, we transplanted wild-type bone marrow cells into VEGFR-$1^{+/-}$ mice or their wild-type littermates. We then produced a hindlimb ischemia model and assessed blood flow recovery and the capillary density of ischemic tissue. Despite the transplantation of wild-type bone marrow, blood flow recovery was still significantly impaired in VEGFR-$1^{+/-}$ mice (Figure 5B). The number of CD31-positive cells was also lower in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5C). Thus, it is unlikely that impaired neovascularization in VEGFR-$1^{+/-}$ mice is attributed to reduced migration of bone marrow–derived cells. We could not detect VEGFR-1 expression in muscle cells (supplemental Figure IV). It was noted that the number of endothelial cells double positive for phospho-Akt and CD31 was significantly higher in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5D).

Inhibition of Akt Signaling Ameliorates the Impairment of Neovascularization in VEGFR-$1^{+/-}$ Mice

Next, we examined whether an increase of endothelial Akt activity contributed to impaired neovascularization in VEGFR-$1^{+/-}$ mice. Akt1 is the predominant isoform of Akt in endothelial cells and is thought to play an important role in postnatal angiogenesis.[30] It has been reported that the angiogenic response of $Akt1^{-/-}$ mice was enhanced in a tumor angiogenesis model, but was decreased in a hindlimb ischemia model,[30,31] so we thus used $Akt1^{+/-}$ mice for our in vivo experiments. Consistent with the previous reports,[32] phospho-Akt levels were lower in the aorta of $Akt1^{+/-}$ mice compared with wild-type littermates (supplemental Figure V). After creating hindlimb ischemia in VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice, we examined the extent of blood flow recovery and the capillary density 1 week later. We found that there were no significant differences of blood flow recovery and capillary density between $Akt1^{+/-}$ mice and $Akt1^{+/+}$ mice (Figure 6A and 6B). Decreased VEGFR-1 expression significantly reduced blood flow recovery in $Akt1^{+/+}$ mice, but not in $Akt1^{+/-}$ mice (Figure 6A). Likewise, the capillary density of ischemic tissue was significantly reduced in VEGFR-$1^{+/-}$ $Akt1^{+/+}$ mice compared with wild-type mice, but VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice had a similar capillary density to that of VEGFR-$1^{+/+}$ $Akt1^{+/-}$ mice (Figure 6B). These results suggest that an increase of endothelial Akt activity may be responsible for impaired neovascularization in VEGFR-$1^{+/-}$ mice.

Discussion

In the present study, we demonstrated that VEGFR-1 modulates postnatal angiogenesis through inhibition of the excessive activation of Akt by VEGF. It has been reported that VEGF and VEGFR-1 can be simultaneously induced by various stimuli, including hypoxia.[33] Thus, the role of VEGFR-1 may vary, depending on the extent of activation of Akt. For example, when overproduction of growth factors such as VEGF and insulin leads to excessive activation of Akt and impairs normal regulation of endothelial proliferation,

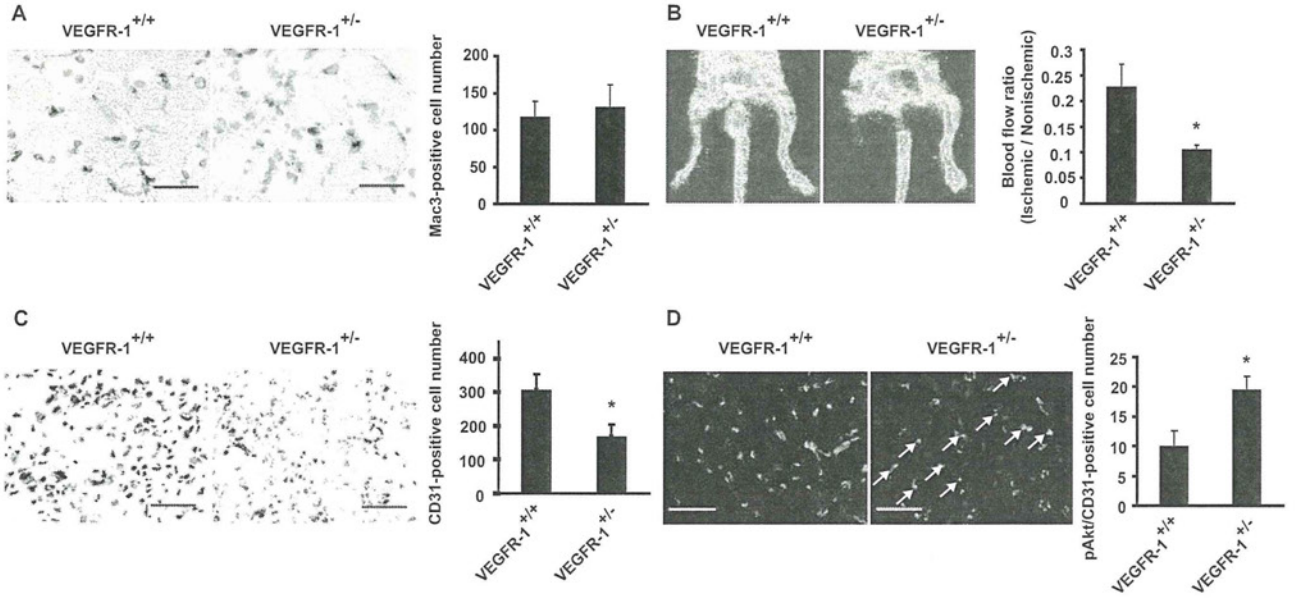

Figure 5. Role of bone marrow–derived cells in impaired neovascularization in VEGFR-1$^{+/-}$ mice. A, Immunohistochemistry for Mac3 (brown) in ischemic limbs. Scale bar: 50 μm. The number of Mac3-positive cells per square millimeter is shown (n=4). B, Wild-type bone marrow cells were transplanted into VEGFR-1$^{+/-}$ mice or their wild-type littermates. Limb perfusion was measured by a laser Doppler analyzer at 1 week after ischemia. *P<0.05 vs wild-type littermates (n=6). C, Immunohistochemistry for CD31 (brown) in ischemic limbs of bone marrow–transplanted mice. Scale bar: 50 μm. *P<0.05 s wild-type littermates (n=6). D, Activation of Akt in endothelial cells of ischemic limbs from VEGFR-1$^{+/-}$ mice. Representative immunostainings for phospho-Akt (red) and CD31 (green) were shown. Arrows indicate phospho-Akt/CD31-positive cells (yellow). Scale bar: 50 μm. The graph shows the ratio of phospho-Akt/CD31-positive cell number to all CD31-positive cell number. *P<0.05 vs wild-type littermates (n=5).

VEGFR-1 may act as a positive regulator of angiogenesis by inhibiting activation of VEGFR-2. Conversely, VEGFR-1 may exert a negative effect on angiogenesis when growth factors appropriately activate the Akt signaling pathway to induce endothelial cell proliferation. These mechanisms may provide an explanation as to why the effects of PlGF on angiogenesis were reported to differ.

Although there is evidence to suggest that VEGFR-1 interacts with the p85 subunit of phosphatidylinositol-3 kinase (PI3K) to regulate its activity,[34–36] VEGFR-1 appears to exert its inhibitory effect on angiogenesis mainly by blocking the activation of Akt mediated by VEGF via VEGFR-2 for the following reasons. First, treatment with VEGF-A increased Akt activity in VEGFR-1–deleted cells, but not in VEGFR-2–deleted cells (Figure 3A and 3B). Second, treatment with a neutralizing anti-VEGF antibody reduced the enhanced activation of Akt in VEGFR-1-deleted cells (Figure 3C). Finally, treatment with PlGF did not provoke any

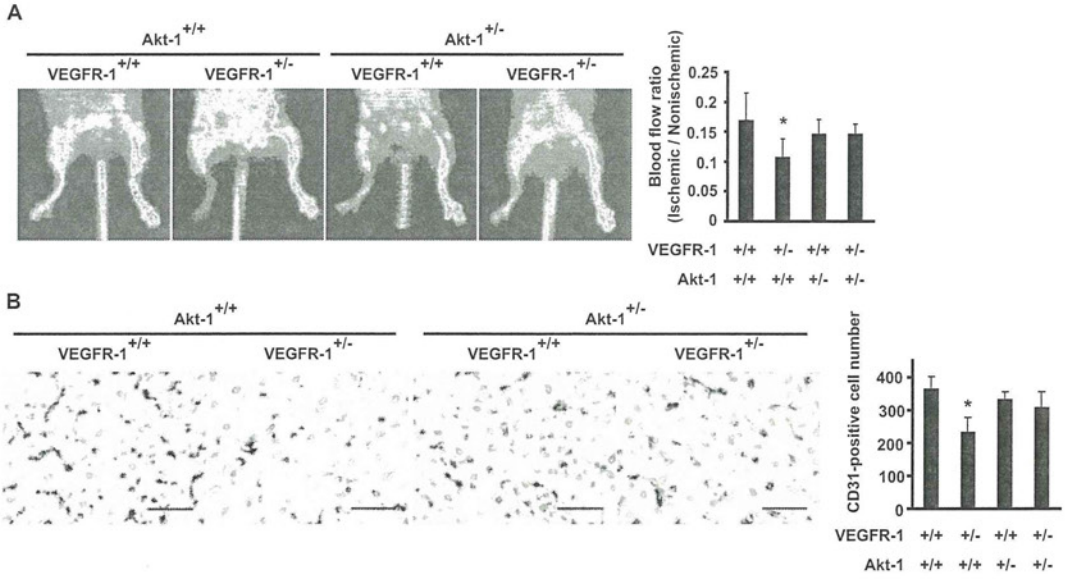

Figure 6. Inhibition of Akt signaling ameliorates the impairment of neovascularization in VEGFR-1$^{+/-}$ mice. A, Limb perfusion was measured by a laser Doppler analyzer at 1 week after creation of ischemia. *P<0.01 vs wild-type littermates (n=14 to 18). B, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. *P<0.05 vs wild-type littermates (n=6 to 7).

biological response in the presence of anti-VEGF antibody (J. Nishi, T. Minamino, unpublished data, 2007). Our results are consistent with previous studies[37,38] demonstrating that tyrosine phosphorylation of VEGFR-2 was elevated in VEGFR-1–deficient embryonic stem cells, whereas loss of VEGFR-1 led to decreased sprout formation and migration, which resulted in reduced vascular branching. This reduction was restored by blockade of the VEGFR-2 signaling pathway as well as by treatment with soluble VEGFR-1. Although Bussolati et al demonstrated that VEGFR-1 but not VEGFR-2 increases endothelial production of NO, thereby promoting tube formation,[39] cGMP production was significantly decreased in VEGFR-1–deleted endothelial cells (supplemental Figure ID). Moreover, VEGF treatment failed to activate Akt in VEGFR-2-deleted endothelial cells (Figure 3B) and introduction of mutant VEGFR-1 lacking the sites for interaction with PI3K did not mimic the effects of shVEGFR-1 (J. Nishi, T. Minamino, unpublished data, 2007). Taken together, these results suggest that VEGFR-1 acts to provide "fine tuning" of VEGF signaling to achieve the proper formation of blood vessels. The biological consequences of VEGFR-1 deletion appears to be related to loss of its decoy effect, but other mechanisms might be involved such as "cross talk" between VEGFR-1 and VEGFR-2,[8,16,17] direct regulation of the VEGFR-2 signaling pathway by VEGFR-1,[39,40] and some undefined effect of the extracellular domain of membrane-bound VEGFR-1.[41]

We have previously demonstrated that constitutive activation of Akt induced by insulin promotes senescence-like arrest of endothelial cell growth via a p53/p21-dependent pathway.[19] Moreover, tube formation was significantly reduced by overactivation of Akt. Likewise, constitutive activation of Akt has been reported to promote the senescence in other types of cells such as endothelial progenitors and mouse embryonic fibroblasts.[42,43] The study using conditional transgenic mice has demonstrated that sustained activation of Akt in endothelial cells causes increased blood vessel size and generalized edema within 2 weeks and that these changes are reversible.[44] Using the same mouse model, it has been reported that chronic activation of Akt over 8 weeks leads to endothelial cell senescence and loss of endothelium-dependent stroke protection.[45] Recent studies by several groups demonstrated that diabetic state induces activation of the Akt pathway, thereby contributing to the pathology of diabetic complications.[42,46–48] We also detected increased Akt activity in endothelial cells on the surface of coronary atherosclerotic lesions in patients with diabetes.[19] Moreover, accumulating evidence suggests that vascular cell senescence contributes to the pathogenesis of age-associated vascular diseases including diabetic vasculopathy.[49] Thus, these results suggest the potential of the treatment for vascular dysfunction associated with diabetes and aging by modulating Akt activity with a soluble form of VEGFR-1.

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Disclosures

None.

References

1. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005; 438:932–936.
2. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669–676.
3. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438:937–945.
4. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66–70.
5. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development*. 1999;126:3015–3025.
6. Kearney JB, Ambler CA, Monaco KA, Johnson N, Rapoport RG, Bautch VL. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood*. 2002;99:2397–2407.
7. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A*. 1998;95:9349–9354.
8. Rahimi N, Dayanir V, Lashkari K. Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells. *J Biol Chem*. 2000;275: 16986–16992.
9. Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, Ferrara N. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem*. 1996;271: 5638–5646.
10. Yang S, Xin X, Zlot C, Ingle G, Fuh G, Li B, Moffat B, de Vos AM, Gerritsen ME. Vascular endothelial cell growth factor-driven endothelial tube formation is mediated by vascular endothelial cell growth factor receptor-2, a kinase insert domain-containing receptor. *Arterioscler Thromb Vasc Biol*. 2001;21:1934–1940.
11. Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem*. 2001;276:3222–3230.
12. Errico M, Riccioni T, Iyer S, Pisano C, Acharya KR, Persico MG, De Falco S. Identification of placenta growth factor determinants for binding and activation of Flt-1 receptor. *J Biol Chem*. 2004;279:43929–43939.
13. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8:831–840.
14. Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res*. 2002;62:2749–2752.
15. Odorisio T, Schietroma C, Zaccaria ML, Cianfarani F, Tiveron C, Tatangelo L, Failla CM, Zambruno G. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J Cell Sci*. 2002;115:2559–2567.
16. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dom-

browski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*. 2003;9:936–943.
17. Neagoe PE, Lemieux C, Sirois MG. Vascular endothelial growth factor (VEGF)-A165-induced prostacyclin synthesis requires the activation of VEGF receptor-1 and -2 heterodimer. *J Biol Chem*. 2005;280: 9904–9912.
18. Shih SC, Ju M, Liu N, Smith LE. Selective stimulation of VEGFR-1 prevents oxygen-induced retinal vascular degeneration in retinopathy of prematurity. *J Clin Invest*. 2003;112:50–57.
19. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *Embo J*. 2004;23:212–220.
20. O'Neill BT, Abel ED. Akt1 in the cardiovascular system: friend or foe? *J Clin Invest*. 2005;115:2059–2064.
21. Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, Risau W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem*. 1996;271: 17629–17634.
22. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood*. 1996;87:3336–3343.
23. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood*. 2001;97:785–791.
24. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. 2001;7:1194–1201.
25. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med*. 2002;8:841–849.
26. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat Med*. 2006; 12:557–567.
27. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 2000;6:389–395.
28. Hiratsuka S, Maru Y, Okada A, Seiki M, Noda T, Shibuya M. Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. *Cancer Res*. 2001;61: 1207–1213.
29. Murakami M, Iwai S, Hiratsuka S, Yamauchi M, Nakamura K, Iwakura Y, Shibuya M. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages. *Blood*. 2006;108:1849–1856.
30. Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med*. 2005;11:1188–1196.
31. Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC. Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J Clin Invest*. 2005;115:2119–2127.
32. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev*. 2001;15:2203–2208.
33. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med*. 2003;9:677–684.
34. Cunningham SA, Waxham MN, Arrate PM, Brock TA. Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. *J Biol Chem*. 1995;270:20254–20257.
35. Igarashi K, Isohara T, Kato T, Shigeta K, Yamano T, Uno I. Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. *Biochem Biophys Res Commun*. 1998;246:95–99.
36. Yu Y, Hulmes JD, Herley MT, Whitney RG, Crabb JW, Sato JD. Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediates phosphatidylinositol 3′-kinase binding. *Biochem J*. 2001;358:465–472.
37. Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *Am J Pathol*. 2004;164:1531–1535.
38. Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood*. 2004;103: 4527–4535.
39. Bussolati B, Dunk C, Grohman M, Kontos CD, Mason J, Ahmed A. Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am J Pathol*. 2001;159:993–1008.
40. Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 downmodulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem*. 2001;276:26969–26979.
41. Hiratsuka S, Nakao K, Nakamura K, Katsuki M, Maru Y, Shibuya M. Membrane fixation of vascular endothelial growth factor receptor 1 ligand-binding domain is important for vasculogenesis and angiogenesis in mice. *Mol Cell Biol*. 2005;25:346–354.
42. Rosso A, Balsamo A, Gambino R, Dentelli P, Falcioni R, Cassader M, Pegoraro L, Pagano G, Brizzi MF. p53 Mediates the accelerated onset of senescence of endothelial progenitor cells in diabetes. *J Biol Chem*. 2006;281:4339–4347.
43. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005;436:725–730.
44. Phung TL, Ziv K, Dabydeen D, Eyiah-Mensah G, Riveros M, Perruzzi C, Sun J, Monahan-Earley RA, Shiojima I, Nagy JA, Lin MI, Walsh K, Dvorak AM, Briscoe DM, Neeman M, Sessa WC, Dvorak HF, Benjamin LE. Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell*. 2006;10:159–170.
45. Wang C, Kim H, Hiroi Y, Mukai Y, Satoh M, Liao JK. Increase cellular senescence and cerebral infarct size in mice with chronic activation of endothelial protein kinase Akt. *Circulation*. 2006;114:II-160.
46. Hojlund K, Staehr P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JF. Increased phosphorylation of skeletal muscle glycogen synthase at NH2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes*. 2003;52:1393–1402.
47. Sheu ML, Ho FM, Yang RS, Chao KF, Lin WW, Lin-Shiau SY, Liu SH. High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. *Arterioscler Thromb Vasc Biol*. 2005;25:539–545.
48. Clodfelder-Miller B, De Sarno P, Zmijewska AA, Song L, Jope RS. Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. *J Biol Chem*. 2005;280:39723–39731.
49. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. *Circ Res*. 2007;100:15–26.

Supplementary Material and Methods

Short hairpin interference RNA Vectors

The mammalian retrovirus expression vector pSIREN-RetroQ (Clontech) was used to achieve the expression of short hairpin interference RNA (shRNA) in human endothelial cells. The following inserts were employed: VEGFR-2; shVEGFR-2-a (5'-ggatgaacattgtgaacga-3') and shVEGFR-2-b (5'- gctcctgaagatctgtata-3'), VEGFR-1; shVEGFR-1-a (5'-gagcaaacgtgacttattt-3') and shVEGFR-1-b (5'-gcagcacgctgtttattga-3'). Each sequence was separated by a 9-nucleotide noncomplementary spacer from the reverse complement of the same 19-nucleotide sequence. The 9-nucleotide noncomplementary spacer sequence used for hairpin loop formation was 5'-TTCAAGAGA-3', except for shVEGFR-1-a. The 9-nucleotide noncomplementary spacer sequence for shVEGFR-1-a was 5'- AAGTTCTCT-3'. The non-silencing control insert was 5'-GTGCGTTGCTAGTACCAACTTCAAGAGA-3', which was designed by the manufacturer and showed no significant homology with any mammalian gene sequence (shNega).

Retroviral infection

The plasmids used for generating retroviruses were pSiren RetroQ (Clontech) and pLNCX (Clontech). We created a pLNCX-based vector expressing a dominant-negative form of Akt (DN-Akt), wild-type VEGFR-1, or E6 oncoprotein. Retroviral stock solutions were generated by transient transfection of a packaging cell line (PT67, Clontech) and were stored at –80°C until use. Human endothelial cells (at passages 4–6) were plated at 5×10^5 cells in 100-mm dishes at 24 hours before infection. Then the culture medium was replaced by a retroviral stock solution supplemented with 8 μg/ml polybrene (Sigma, Tokyo, Japan). After 48 hours, the infected cell population

was selected by culture for 4 days in 0.8 μg/ml puromycin (pSiren RetroQ-based vectors) or by culture for 7 days in 500 μg/ml G418 (pLNCX-based vectors). After selection, 2×10^5 cells were seeded into 100-mm dishes on the 5th day following infection (which was designated as day 0). For double infection, cells were infected with pLNCX or pLNCX-DN-Akt, purified with 500 μg/ml G418 for 7 days, and then subjected to repeat infection with pSiren RetroQ-based vectors as described above. We confirmed that retroviral infection of shVEGFR-1/2 caused stable down-regulation of VEGFR-1/2 during the long-term culture.

Cell culture

Recombinant human VEGF165 (293-VE), recombinant human PlGF (264-PG-010), and monoclonal anti-human VEGF antibody (MAB293) were purchased from R & D Systems (Minneapolis, MN). Human umbilical vein endothelial cells were purchased from Cambrex (Walkersville, MD), and were cultured according to the manufacturer's instructions. Endothelial cell proliferation was assessed by determining cell counts after culture in the presence of VEGF-A (10 ng/ml) for 3 days. For the long-term culture, the cells were trypsinized, counted using trypan blue exclusion, and subcultured at a density of 3,500 cells/cm^2 whenever the culture became nearly confluent. The cultures were terminated and regarded as senescent when the cell population did not increase in 2 weeks. We confirmed the senescent phenotype by assay of senescence-associated β-galactosidase activity. Senescence-associated β-galactosidase assay was performed as described previously[1]. Population doublings (PD) were calculated as follows: PD = log (number of cells obtained/initial number of cells)/log 2.

Western blot analysis

Whole cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 5 mM EDTA, 0.025% NaN3, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, and 1 mM Na_2VO_3). The lysates (30 μg) were resolved by SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and incubated with the primary antibody followed by anti-rabbit immunoglobulin G-horseradish peroxidase antibody or anti-mouse immunoglobulin G-horseradish peroxidase antibody (Jackson, West Grove, PA). Specific proteins were detected by using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The primary antibodies used for Western blotting were as follows:

anti-pERK antibody (sc-7383), anti-p53 antibody (DO-1), anti-actin antibody (sc-8432), anti-β tubulin antibody (sc-5274), anti-VEGFR-2 antibody (sc-6251), anti-bax antibody (sc-493), anti-bcl-2 antibody (sc-492), anti-eNOS antibody (sc-654) (Santa Cruz, Santa Cruz, CA); anti-p21 antibody (Oncogene, Cambridge, MA); anti-phospho-Akt antibody (Ser473, #9271), anti-cleaved caspase 3 antibody, anti-phospho-eNOS antibody (Cell Signaling, Beverly, MA); anti-actin antibodies (A-5316, A-5060), and anti-VEGFR-1 antibody (V4262) (Sigma). Because Akt activity is significantly affected by food intake, all mice were subjected to over-night starvation before sacrifice. The samples were then subjected to Western blotting for phospho-Akt.

Northern blotting

Total RNA (30 μg) was extracted by the guanidinium thiocyanate-phenol chloroform method using RNA zol B (Tel Test, Friendswood, TX) according to the manufacturer's instructions, separated on formaldehyde denaturing gel, and transferred to a nylon membrane (Amersham, Bucks, UK). The blot was then hybridized with radiolabeled

VEGFR-2[2] or VEGFR-1 cDNA probes (a kind gift from Dr Thomas F. Zioncheck at Genentech Inc., CA) using Quickhyb hybridization solution (Stratagene, Tokyo, Japan) according to the manufacturer's instructions.

Luciferase assay

After infection with shNega, shVEGFR-1, or shVEGFR-2, endothelial cells were seeded at a density of 3×10^4 cells in 35-mm dishes and cultured for 24 hours in growth medium (EGM2). Then we transfected the reporter gene plasmid (2 μg) into the infected endothelial cells and continued culture in growth medium (EGM2) for 12 hours. Next, we replaced the growth medium with serum-supplemented basal medium (EBM2 containing 5% serum, heparin, ascorbic acid, and hydrocortisone) and cultured the cells for 36 hours with VEGF (10 ng/ml). We measured luciferase activity at 48 hours after transfection. A vector encoding Renilla luciferase (0.1 μg) was co-transfected as an internal control. The assay was performed by using a dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. The plasmid pPG13-Luc[3] was a gift from Dr B. Vogelstein (John Hopkins University, Baltimore, MD).

Measurement of cGMP production

Production of cGMP was examined with cGMP ELISA (Cayman Chemical, Cayman) according to manufacturer's instructions.

Animals

Akt1-deficient mice (Akt1$^{+/-}$, in a C56BL6 background) were a kind gift from Dr Morris J Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA). Generation and genotyping of VEGFR-1-deficient mice (VEGFR-1$^{+/-}$, in an ICR background) and Akt1-deficient mice have been described previously[4, 5]. VEGFR-1$^{+/-}$ Akt1$^{+/-}$ mice were obtained by intercrossing VEGFR-1$^{+/-}$ and Akt1$^{+/-}$ mice and the first generation offspring was used for all experiments. All of the mice used in this study were 8 to 12 weeks old. All experimental procedures were performed according to the guidelines for animal experiments of Chiba University, and the protocols were approved by our institutional review board.

Hindlimb ischemia model

After mice were anesthetized, the proximal part of the femoral artery and the distal portion of the popliteal artery were ligated and stripped out after all side branches were dissected free. After 7 days, hindlimb perfusion was measured with a laser Doppler perfusion analyzer (Moor Instruments, Devon, UK). To determine whether non-endothelial cells express VEGFR-1 in hind limbs, we utilized VEGFR-1-deficient mice in which the Lac Z gene is inserted into the VEGFR-1 gene locus. As shown in Supplementary Figure, the Lac Z activity was detected only in endothelial cells.

Histology

Gastrocnemius and soleus muscle tissues were removed from the ischemic limbs after systemic perfusion with PBS and were immediately embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan). Each specimen was snap frozen in liquid

nitrogen and cut into 4 μm sections. The sections were fixed in acetone, stained with an antibody for CD31 or Mac 3 (Pharmingen, San Diego, CA), and counterstained with hematoxylin. Then two transverse sections of the gastrocnemius and soleus muscle were photographed digitally at a magnification of ×100 (15-20 photographs per mouse). Capillary endothelial cells and macrophages were quantified as the number per square millimeter. When immunostaining was done for phospho-Akt (Santa Cruz), the mice were fasted for 24 hours before sacrifice to avoid the influence of feeding. To assess the level of Akt activity in endothelial cells, phospho-Akt/CD31 double-positive cells were counted.

Tube-forming assay

The tube-forming assay was performed according to the manufacturer's instructions (Bio Coat Angiogenesis System, Clontech) with minor modifications. Human endothelial cells were seeded in 96-well plates at a density of 1.0×10^4 cells in the serum-free basic medium EBM2 (Cambrex) containing heparin, ascorbic acid and hydrocortisone with or without VEGF (50 ng/ml). After 16 hours, capillary-like tube formation was assessed by photography under an inverted phase contrast microscope at 25× magnification. The total tube length was estimated by using an angiogenesis image analyzer (Kurabo, Osaka, Japan). This assay was performed in triplicate.

Cell death assay

Endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2 were seeded at a density of 1×10^5 cells in 60-mm dishes and cultured for 24 hours in growth medium (EGM2, Cambrex). After washing the cells twice with PBS, serum-free DMEM with

or without VEGF (10 ng/ml) was added. After 24 hours of serum starvation, both detached and attached cells were collected and stained with 0.2% trypan blue (Sigma), and the number of viable cells was counted with a hemocytometer. Apoptosis was assessed by quantification of cleaved caspase 3 in whole cell lysates (30 μg) by Western blot analysis.

Bone marrow transplantation model

Before bone marrow transplantation, 8 to 10-week-old male VEGFR-$1^{+/-}$ mice or their wild-type littermates were exposed to total body irradiation (9 mGy). Bone marrow cell suspensions were isolated by flushing the femurs and tibias harvested from wild-type mice. Bone marrow cell suspensions (1.5×10^7 cells) were injected intravenously via the tail vein within 6 hours of irradiation. The chimeric rate was higher than 95% as determined by FACS analysis of chimeric mice transplanted with bone marrow cells from the green fluorescent protein (GFP) transgenic mice.

References

1. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *Embo J.* 2004;23:212-220.
2. Sawano A, Takahashi T, Yamaguchi S, Aonuma M, Shibuya M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. *Cell Growth Differ.* 1996;7:213-221.
3. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 1993;75:817-825.

4. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* 1995;376:66-70.

5. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem.* 2001;276:38349-38352.

Supplementary Figure Legends

Online Figure I. Effect of VEGF receptor gene silencing on endothelial cell function.

(A) Human umbilical vein endothelial cells were infected with retroviral vectors expressing a short hairpin interference RNA that targeted the VEGFR-2 gene (shVEGFR-2a and shVEGFR-2b) or the VEGFR-1 gene (shVEGFR-1a and shVEGFR-1b), and then the cells were purified by culture with antibiotics. shNega served as the control vector. Whole cell lysates (30 μg) was extracted from infected cells and analyzed to assess the expression of VEGFR-1 or VEGFR-2 by Western blot analysis. Actin served as the loading control.

(B) Whole cell lysates (30 mg) were prepared as described in legend for Figure 2B and analyzed to investigate Bax and Bcl-2 expression by Western blot analysis.

(C) Infected cells were cultured in serum-free basal medium (without VEGF-A) for 8 hours and subsequently treated with VEGF-A (10 ng/ml) for 5-30 minutes. Whole cell lysates were extracted at indicated times and phospho-eNOS expression was investigated by Western blot analysis. eNOS served as the control (lower panel).

(D) Cell lysates were extracted from endothelial cells infected with shVEGFR-1 or shNega in the presence of VEGF-A (10 ng/ml) and cGMP production was measured. $p^{*}<0.05$ vs. shNega (n=3).

Online Figure II. Inhibition of Akt activation suppresses induction of p21 expression.

Human endothelial cells were infected with pLNCX (Mock) or pLNCX DN-Akt (DN-Akt). Infected cell populations were then transduced with shNega or shVEGFR-1. Whole cell lysates (30 μg) were obtained from double-infected

endothelial cells and p21 expression was assessed by Western blot analysis. Similar results were obtained from three independent experiments.

Online Figure III. Phopho-Akt levels in VEGFR-1$^{+/-}$ mice.

Phopho-Akt (pAkt) and Akt levels were examined in the aorta of VEGFR-1$^{+/-}$ mice (VEGFR-1$^{+/-}$) and wild-type littermates (VEGFR-1$^{+/+}$) by Western blot analysis. $p^*<0.05$ vs. VEGFR-1$^{+/+}$ (n=4).

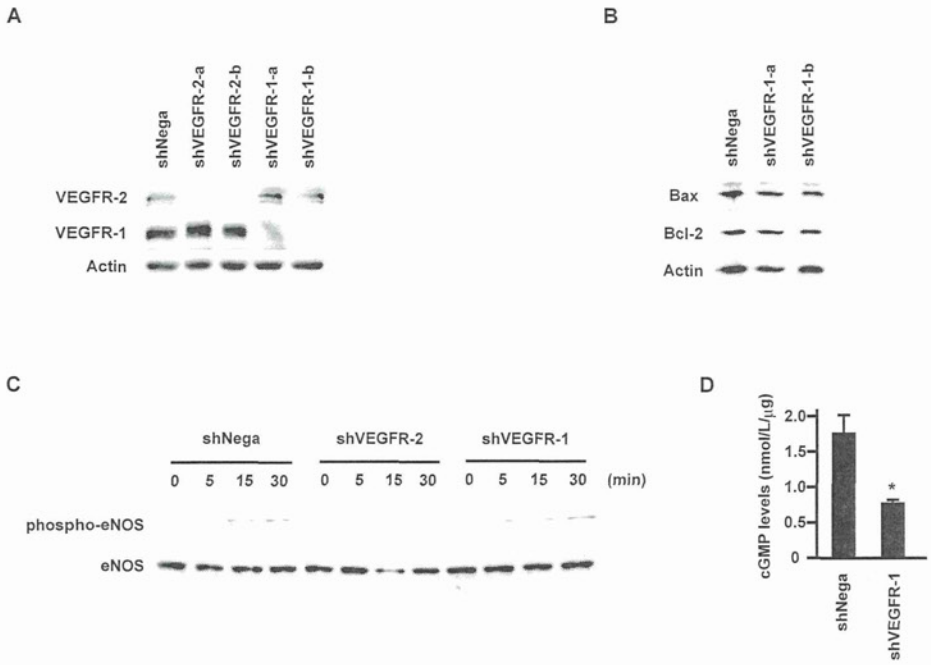
Online Figure IV. Expression of VEGFR-1 in hind limbs.

In VEGFR-1-deficient mice, the Lac Z gene is inserted into the VEGFR-1 gene locus. VEGFR-1$^{+/-}$ mice were subjected to β-galactosidase activity assay to locate endogenous VEGFR-1 expression in hind limbs. Scale bar: 50 μm

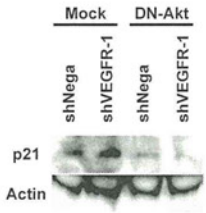
Online Figure V. Phopho-Akt levels in Akt-1$^{+/-}$ mice.

Phopho-Akt (pAkt) and Akt levels were examined in the aorta of Akt-1$^{+/-}$ mice (Akt-1$^{+/-}$) and wild-type littermates (Akt-1$^{+/+}$) by Western blot analysis.

Online Figure I



Online Figure II



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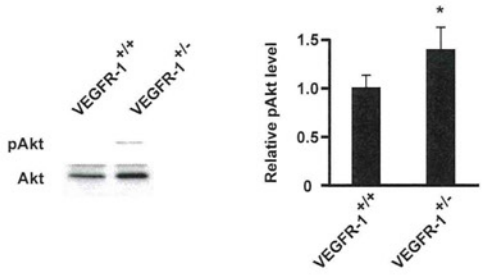
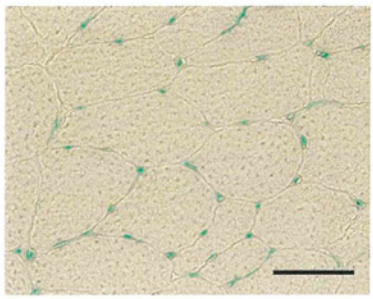


Online Figure IV



Online Figure V