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Research Article **Vascular biology**

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Akt1/protein kinase Bα is critical for ischemic and VEGF-mediated angiogenesis

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Akt, or protein kinase B, is a multifunctional serine-threonine protein kinase implicated in a diverse range of cellular functions including cell metabolism, survival, migration, and gene expression. However, the in vivo roles and effectors of individual Akt isoforms in signaling are not explicitly clear. Here we show that the genetic loss of Akt1, but not Akt2, in mice results in defective ischemia and VEGF-induced angiogenesis as well as severe peripheral vascular disease. Akt1 knockout (*Akt1*$^{-/-}$) mice also have reduced endothelial progenitor cell (EPC) mobilization in response to ischemia, and reintroduction of WT EPCs, but not EPCs isolated from *Akt1*$^{-/-}$ mice, into WT mice improves limb blood flow after ischemia. Mechanistically, the loss of Akt1 reduces the basal phosphorylation of several Akt substrates, the migration of fibroblasts and ECs, and NO release. Reconstitution of *Akt1*$^{-/-}$ ECs with Akt1 rescues the defects in substrate phosphorylation, cell migration, and NO release. Thus, the Akt1 isoform exerts an essential role in blood flow control, cellular migration, and NO synthesis during postnatal angiogenesis.

Introduction

Many substances such as growth factors, bioactive lipids, statin-based drugs, and mechanical forces (shear stress and cyclic strain) can promote angiogenesis and signal via activation of the PI3K/Akt pathway in cultured ECs (1–7). Activation of the PI3K/Akt pathway accounts for many of the actions of angiogenic growth factors such as VEGF, including cell survival, migration, tube formation, and promotion of the release of NO. Evidence supporting the role of PI3K/Akt in such pathways in ECs includes blockade of PI3K with inhibitors and overexpression of dominant-negative constructs for Akt; however, there is little direct genetic evidence supporting a role for Akt in regulating angiogenesis in vivo. Surprisingly, Akt1 and Akt2 are not essential for embryonic vasculogenesis since Akt1- and Akt2-deficient mice are viable (8–10). However, *Akt1*$^{-/-}$ mice have smaller litter sizes, impaired extraembryonic vascular patterning and placental hypotrophy, reduced fetal weight, and a partially penetrant phenotype of a higher fetal mortality. The defects in placental architecture and angiogenesis have been associated with a decrease in eNOS phosphorylation but not a decrease of eNOS or VEGF levels (11). The overwhelming dogma supporting an essential role for Akt in mediating the actions of many proangiogenic factors in cultured ECs is at odds with the lack of an embryonic or adult vascular phenotype in Akt1-deficient mice, suggesting several possibilities including the following: (a) gene compensation by additional Akt isoforms or pathways occurs during development; (b) signaling pathways discovered in cultured ECs may not be relevant to in vivo angiogenesis; or (c) embryonic and postnatal angiogenesis are governed by overlapping but distinct signaling systems that may fine-tune specialized functions in either the developing embryo or the adult.

In order to directly explore the roles of Akt isoforms during postnatal angiogenesis in the adult, we used mice deficient in either Akt1 (8) or Akt2 (9) and examined several angiogenic phenotypes in vivo. Here we show that Akt1 and Akt2 are expressed in vascular tissue and cells and that the loss of Akt1, but not Akt2, dramatically impairs ischemia and VEGF-mediated angiogenesis in vivo and EC functions in vitro. Thus, our data establish Akt1 as a key regulator of postnatal angiogenesis and provide a salient example of the complexity of distinct signaling pathways regulating different forms of angiogenesis.

Results

Expression of Akt1 and Akt2 in cardiovascular tissues. As shown in Figure 1A, when we used mouse lung fibroblasts isolated from F2 generation WT littermates and Akt1- or Akt2-homozygous null mice, RT-PCR revealed the loss of the respective isoform in the appropriate knockout strain with no compensatory changes in the other isoform. Western blot analysis of Akt1 and Akt2 in the heart (Figure 1B, left panel) or gastrocnemius muscle (right panel) confirmed the loss of Akt1 and Akt2 protein expression in the respective knockouts. Next, we examined the distribution of Akt1 and Akt2 proteins in blood vessels isolated from the mice. As shown in Figure 1C, both Akt1 and Akt2 proteins were found in all blood vessels isolated from WT mice, including the aorta, superior mesenteric artery, femoral artery, carotid artery, and jugular vein. Thus, both Akt1 and Akt2 were present in all tissues and isolated vessels examined. Examination of the total Akt phosphorylation on serine 473 and threonine 308 (phosphorylated AktS473

Nonstandard abbreviations used: Ac-Dil-LDL, dioctadecyl-3,3,3′,3′-tetrametylindocarbocyanine perchlorate–labeled acetylated LDL; Ad–β-gal, control virus expressing β-gal; Ad-VEGF, adenovirus expressing murine VEGF 164; EGM-2, endothelial growth medium–2; EPC, endothelial progenitor cell; FKHR, Forkhead in rhabdomyosarcoma; GSK3β, glycogen synthase kinase 3β; Hsp90, heat shock protein 90; MASMC, mouse aortic smooth muscle cell; MDM2, mouse double minute 2; MLEC, mouse lung EC; MLF, mouse lung fibroblast; p-, phosphorylated; rc, reconstituted with.

Conflict of interest: The authors have declared that no conflict of interest exists.

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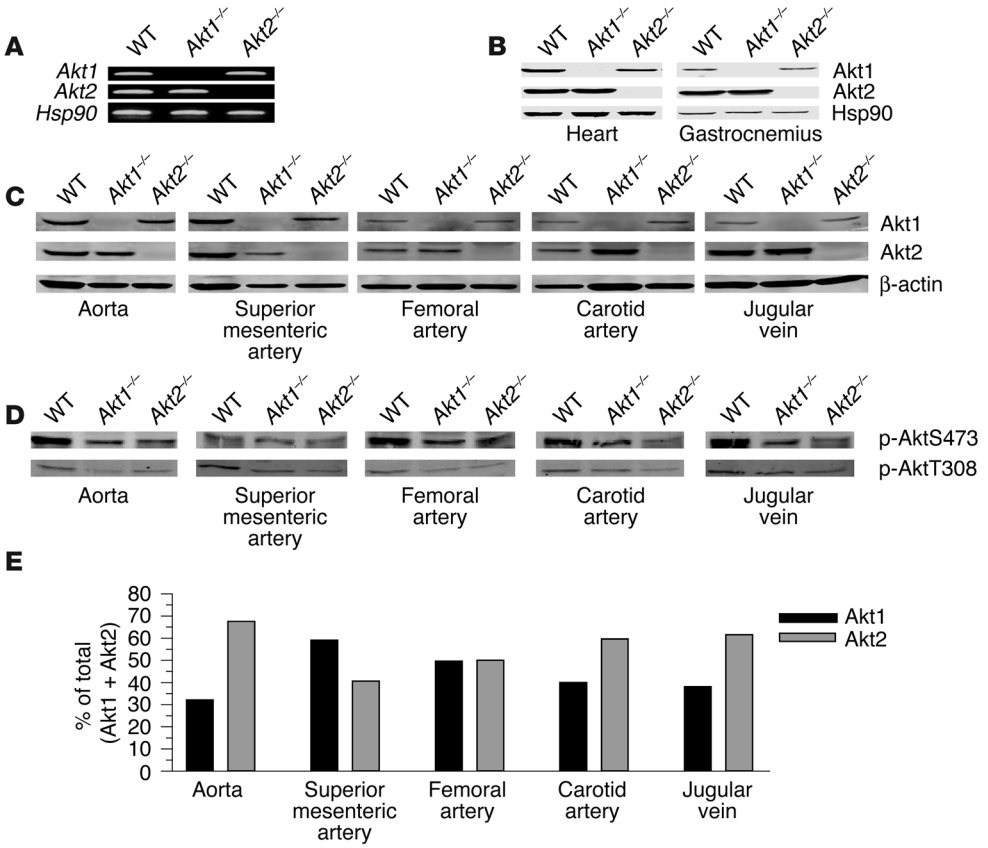

Figure 1
Characterization of tissue and vascular expression of Akt1 and Akt2. (**A**) RT-PCR analysis of RNA isolated from mouse lung fibroblasts using *Akt1*-, *Akt2*-, and *Hsp90*-specific primers. (**B**) Expression of Akt1 and Akt2 protein in homogenates from heart and gastrocnemius muscle by Western blot with antibodies against Akt1, Akt2, or Hsp90 as a loading control. (**C**) Akt1 and Akt2 expression in various blood vessels by Western blot using antibodies against Akt1, Akt2, and β-actin as a loading control. (**D**) p-Akt levels in various blood vessels from WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice. Lysates from 3 animals per group were pooled and analyzed by Western blot using p-AktS473– and p-AktT308–specific Akt antibodies. (**E**) Relative expression of Akt isoforms in blood vessels. Lysates from WT mice were analyzed by SDS-PAGE and quantitative Western blot. Relative protein amounts of Akt1 and Akt2 in the vessels were quantified using standard curves obtained by running recombinant mouse Akt1 and Akt2 proteins. Data represent the mean of 2 pooled samples; $n = 3$.

[p-AktS473] and p-AktT308, respectively) in lysates prepared from the above vessels showed that the loss of either Akt1 or Akt2 reduced total p-Akt levels in the vessel wall (Figure 1D). Since the vessel wall reflects 3 anatomical layers (intima, media, and adventitia), the relative distribution of Akt1 and Akt2 throughout these layers is not known. To determine the relative expression of the Akt isoforms in blood vessels, we performed semiquantitative Western blot analysis on protein samples from the vessels using recombinant, purified murine Akt1, Akt2, and Akt3 as standards (see Methods). As shown in Figure 1E, both Akt1 and Akt2 were differentially expressed in all blood vessels examined, and Akt3 was below the limits of detection.

Akt1 is critical for ischemia-mediated blood flow recovery and angiogenesis. Both in vivo and in vitro data suggest that Akt is critical for angiogenesis since Akt is a key signaling intermediate in response to many angiogenic factors including VEGF (1–3), angiopoietins (4), sphingosine-1-phosphate (5, 6), and statin-based drugs (7) despite the lack of defect in embryonic vasculogenesis (8–11). In order to determine whether Akt influences postnatal angiogenesis, we induced limb ischemia in *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice and examined blood flow, clinical outcome, and indices of angiogenesis. In this model, arteriectomy of the femoral artery diverts blood flow into the internal iliac circulation and elsewhere, which induces arterialization of collateral vessels (arteriogenesis) of the upper limb and increases capillary to skeletal muscle fiber ratio (angiogenesis) in the lower limb. As shown in Figure 2A, the ratio of gastrocnemius blood flow (measured using a deep penetrating laser Doppler probe that determines mean flow of the entire muscle group) of the left limb relative to the right limb before surgery was 1. Following surgery to the left limb, blood flow ratios dropped by 80% in all groups and, in WT and *Akt2*$^{-/-}$ mice, recovered in a time-dependent manner consistent with robust vascularization. However, the loss of Akt1 dramatically impaired lower-limb blood flow recovery at 2 and 4 weeks after surgery, suggesting that Akt1 is essential for postnatal ischemic vascularization. More importantly, the loss of Akt1 was associated with marked limb necrosis as shown in Figure 2B, consistent with a defective recovery in blood flow. Using a clinical scoring system to assess lower-limb function and tissue salvage after surgery (Figure 2C), we determined that the loss of Akt1 was associated with severe tissue ischemia. Next we assessed baseline and ischemia-mediated angiogenesis in the gastrocnemius muscle by quantifying capillary density per muscle fiber. As shown in

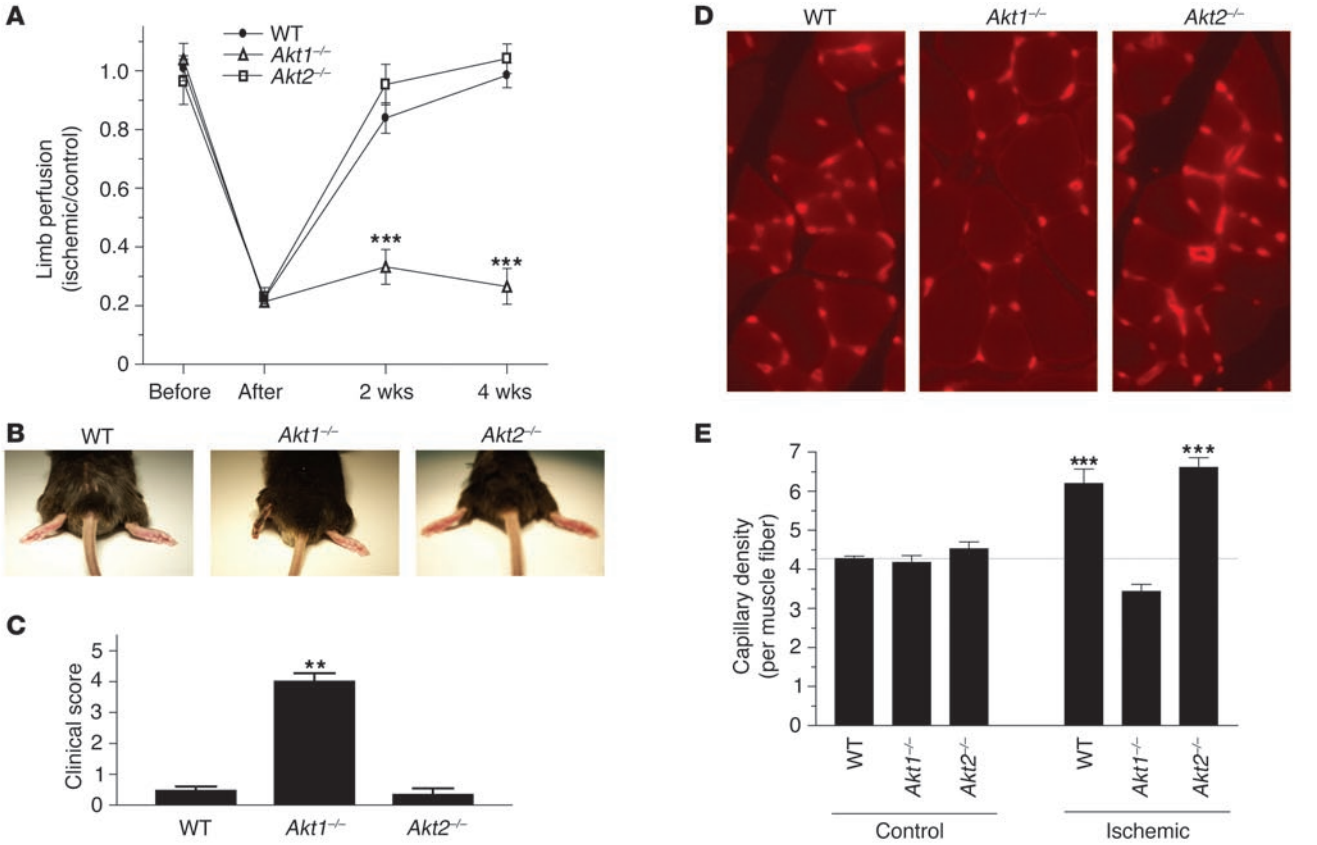

Figure 2

Akt1$^{-/-}$ mice have impaired ischemia-initiated blood flow recovery. (**A**) Blood flow in the gastrocnemius muscle was measured before, immediately after, and at 2 and 4 weeks after left femoral artery resection. Data are expressed as a ratio of the left (ischemic) to right (control) limb perfusion. WT, $n = 14$; *Akt1*$^{-/-}$, $n = 12$; *Akt2*$^{-/-}$, $n = 6$. (**B**) *Akt1*$^{-/-}$ mice developed necrotic toes at 1 week to 2 weeks after left femoral artery resection while *Akt2*$^{-/-}$ and WT littermate mice did not. (**C**) Clinical score at 4 weeks after femoral arteriectomy as an index of severity of limb ischemia: 0, normal; 1, pale foot or gait abnormalities; 2, less than half of foot necrotic; 3, more than half of foot necrotic without lower limb necrosis; 4, more than half of foot necrotic with some lower limb necrosis; 5, necrosis or autoamputation of entire lower limb. WT, $n = 14$; *Akt1*$^{-/-}$, $n = 12$; *Akt2*$^{-/-}$, $n = 6$. (**D**) Representative lectin staining of capillaries from sections of the gastrocnemius/soleus muscles 4 weeks after femoral ligation in WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice. Magnification, ×200. (**E**) Quantification of capillary density, calculated as the number of capillaries per muscle fiber. For each animal, 6–8 randomly selected fields (×200) from 3–4 sections were counted; $n = 5$. $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 2, D and E, baseline capillary densities were identical in WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice. After ischemia, WT and *Akt2*$^{-/-}$ mice showed increased capillary densities whereas *Akt1*$^{-/-}$ mice exhibited reduced angiogenesis (Figure 2E). Thus, despite both Akt1 and Akt2 being present in all vessels examined, we determined that Akt1, but not Akt2, is critical for ischemia-initiated arteriogenesis and angiogenesis.

Ischemia-induced mobilization of endothelial progenitor cells is defective in Akt1$^{-/-}$ *mice.* There is increasing evidence that tissue ischemia induces the emigration of endothelial progenitor cells (EPCs) from the bone marrow into the circulation. These EPCs may home to ischemic tissue and participate in postnatal angiogenesis or remodeling directly by incorporation into the growing vasculature or indirectly through cytokine production (12–14). Therefore we examined ischemia-induced mobilization of EPCs into the circulation from WT or Akt$^{-/-}$ mice. As shown in Figure 3B, baseline EPCs, as isolated from cultured peripheral blood mononuclear cells, were not different in *Akt1*$^{-/-}$ compared with WT mice. Induction of hindlimb ischemia resulted in an increase in EPC mobilization in WT mice, an effect markedly diminished in mice deficient in Akt1 (Figure 3, A and B). Thus, defective EPC mobilization in response to tissue ischemia may contribute to impaired peripheral vascularization and angiogenesis in *Akt1*$^{-/-}$ mice. Next we determined whether EPC-derived Akt1 was critical rescuing limb ischemia. WT mice were rendered ischemic and injected with culture medium or EPCs cultured from the spleens of WT and *Akt1*$^{-/-}$ mice, and blood flow recovery was examined after 2 weeks. As shown in Figure 3C and quantified in Figure 3D, EPCs from WT mice, but not medium or EPCs isolated from *Akt1*$^{-/-}$ mice, improved flow recovery after ischemia. Collectively, these data show that Akt1 is important for both ischemia-induced mobilization and functional responsiveness of EPCs.

VEGF-induced permeability and angiogenesis are impaired in mice lacking Akt1. VEGF is an important angiogenic cytokine that signals via its receptors to activate Akt. VEGF activation of Akt is critical for vessel patterning in zebra fish (15) and vascular development of the chorioallantois in chick embryos (16) and is important for VEGF-mediated survival signals, cell migration and proliferation, and NO release in cultured ECs (3, 17, 18). In order to determine whether Akt1 is necessary for VEGF functions in vivo,

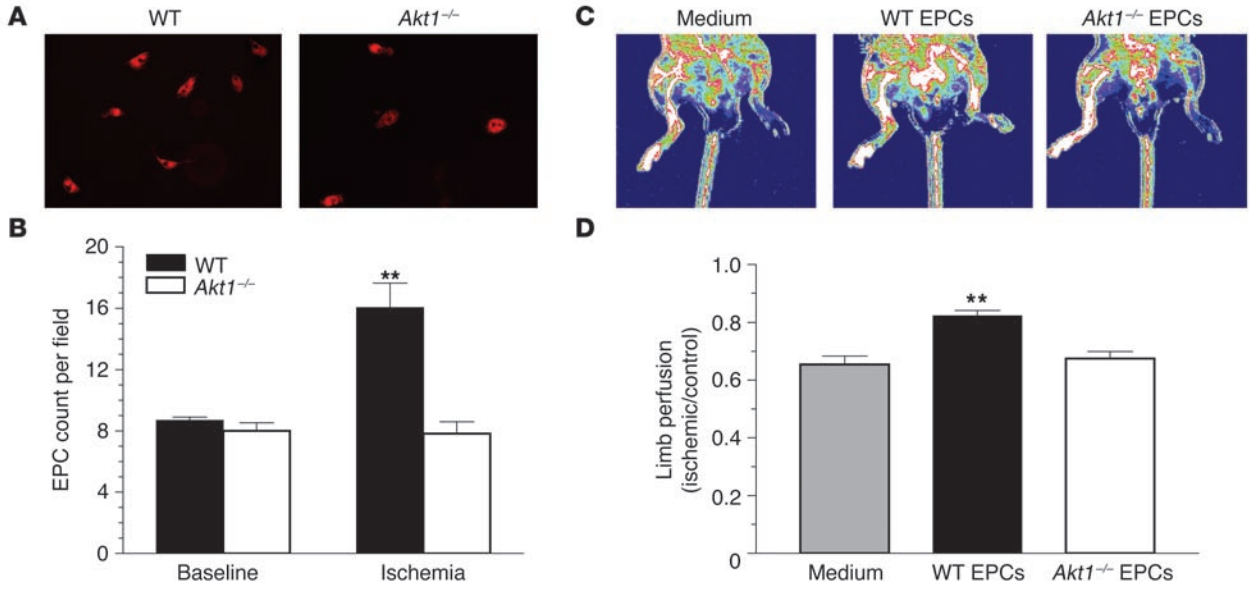

Figure 3
Ischemia-stimulated EPC mobilization is impaired in *Akt1*$^{-/-}$ mice; administration of *Akt1*$^{-/-}$ EPCs does not improve perfusion of ischemic limbs. (**A**) Representative photomicrographs showing Ac-DiI-LDL–positive EPCs isolated from peripheral blood mononuclear cells of WT and *Akt1*$^{-/-}$ mice after femoral ligation. Magnification, ×200. (**B**) For each animal, Ac-DiI-LDL–positive cells in 10 low-power (×100) fields were counted. EPC counts were obtained at baseline and 6 days after induction of limb ischemia; $n = 3–6$. (**C**) Representative laser Doppler blood flow images of the limbs of WT mice at postoperative day 14 after femoral ligation and intravenous administration of medium, WT EPCs, or *Akt1*$^{-/-}$ EPCs (see Methods). (**D**) Quantitative analysis of laser Doppler blood flow images. Data are expressed as a ratio of the left (ischemic) to right (control) limb perfusion; $n = 4$. $^{**}P < 0.01$.

we examined VEGF-induced vascular leakage and angiogenesis, robust bioassays for VEGF action in vivo. For assessment of vascular leakage, WT and *Akt1*$^{-/-}$ mice were intravenously injected with Evans blue, a dye that tightly binds to plasma albumin, and VEGF or saline administered intradermally into the ear. As shown in Figure 4A, WT and *Akt1*$^{-/-}$ mice exhibited similar baseline endothelial barrier function. In contrast, VEGF in WT mice markedly induced vascular leakage after 30 minutes, an effect absent in *Akt1*$^{-/-}$ mice. Next we assessed VEGF-induced angiogenesis, using an intradermal injection of an adenovirus expressing murine VEGF 164 (Ad-VEGF) or a control virus expressing β-gal (Ad–β-gal) into the ears of WT and *Akt1*$^{-/-}$ mice. As shown in Figure 4B, Ad-VEGF increased the number of angiogenic structures (quantified by PECAM-1–positive vascular structures) after 5 days whereas injection of Ad–β-gal did not. In contrast, Ad-VEGF did not promote angiogenesis in *Akt1*$^{-/-}$ mice (Figure 4C). These data demonstrate that *Akt1*$^{-/-}$ mice are resistant to the propermeability and proangiogenic actions of exogenously administered VEGF. Collectively, the above data show that Akt1 is critical for ischemia and VEGF-mediated vascular functions in vivo.

Akt1 regulation of cellular substrates and functions. In order to directly examine cellular and molecular mechanisms that may explain the impaired angiogenic phenotype of the *Akt1*$^{-/-}$ mice, we isolated mouse lung ECs (MLECs) and mouse lung fibroblasts (MLFs) from WT, Akt1, and *Akt2*$^{-/-}$ mice. In addition, we reconstituted *Akt1*$^{-/-}$ cells with retroviruses expressing HA-tagged Akt1 or GFP. As shown in Figure 5A, isolated MLECs expressed endothelial markers PECAM-1 and eNOS, and both MLECs and MLFs (Figure 5, A and B) expressed all 3 Akt isoforms using isoform-selective antibodies. Moreover, the loss of Akt1 or Akt2 did not modulate the expression of the remaining isoforms. As shown in Figure 5C, semiquantitative Western blot analysis of Akt isoform protein expression (using recombinant murine Akt1, Akt2, and Akt3 as standards) in MLECs and mouse aortic smooth muscle cells (MASMCs) showed that Akt1 is the predominant isoform expressed, whereas in MLFs, Akt1 and Akt2 were equally expressed. In all cell types, Akt3 was the least expressed of the Akt isoforms. Comparing data in intact vessels (Figure 1E) and these data from cultured cells, we surmise that Akt1 is the primary isoform in ECs and MASMCs and that Akt2 is the primary isoform in adventitial fibroblasts.

Next, we used phosphospecific antisera to examine the basal phosphorylation of 4 known Akt substrates: eNOS, glycogen synthase kinase 3β (GSK3β), mouse double minute 2 (MDM2), and Forkhead in rhabdomyosarcoma (FKHR; also known as FOXO1). Due to the immortalization procedure using middle T antigen, we could only reliably measure the basal levels of substrate phosphorylation. Middle T antigen strongly activates the PI3K/Akt pathway, making it difficult to reduce basal phosphorylation of Akt substrates with prolonged serum withdrawal (19). As shown in Figure 5D and quantified in Figure 5E, all substrates were basally phosphorylated on their respective Akt phosphorylation sites. The relative increase in FKHR S256 phosphorylation could not be compared to total levels of FKHR since several commercially available antibodies did not detect total FKHR in these cells. The loss of Akt1, but not Akt2, markedly reduced the phosphorylation of eNOS, GSK3β, and FKHR but not MDM2. The reduction in substrate phosphorylation was rescued by the reintroduction of Akt1, which proved that a majority of the basal phosphorylation of eNOS, GSK3β, and FKHR on the respective

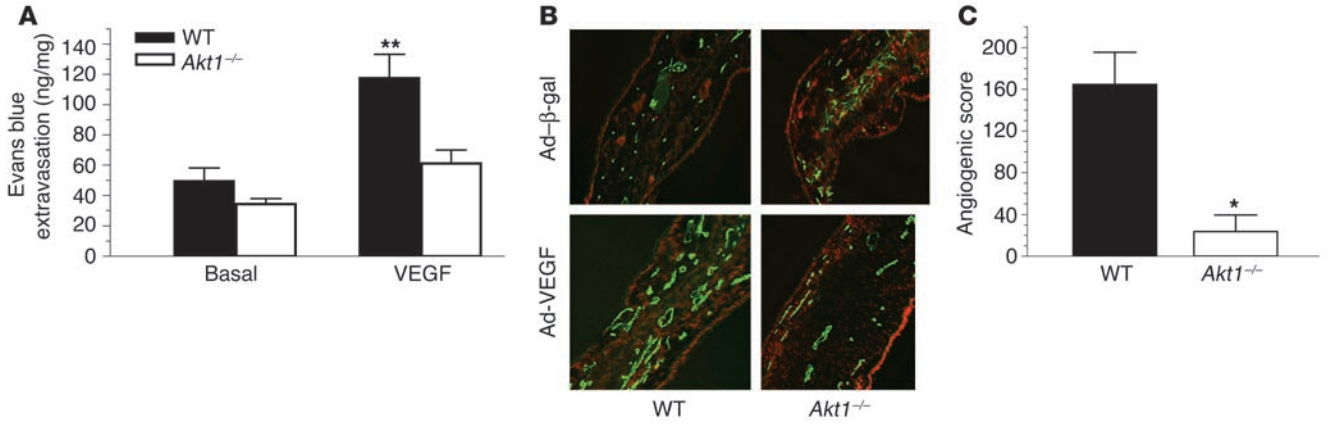

Figure 4

Akt1$^{-/-}$ mice have impaired VEGF-induced vascular permeability and angiogenesis. (**A**) Vascular permeability assessed by Evans blue extravasation 30 minutes after subcutaneous injection of VEGF and saline (Basal) in the right and left ears, respectively. Data are expressed as ng Evans blue per mg dry weight of ear; $n = 7$. (**B**) Representative PECAM-1 staining of frozen ear sections showing impaired VEGF-induced angiogenesis in *Akt1*$^{-/-}$ mice. Ad-VEGF or Ad–β-gal was applied by intradermal injection into the left and right ears, respectively, for 5 days. Frozen sections (7 μm thick) were stained for vessels with anti–PECAM-1 antibody and Alexa 488–conjugated secondary antibody (green). Tissues were counterstained with propidium iodide (red). Magnification, ×100. (**C**) Quantification of PECAM-1 staining. For each animal, 10 low-power (×100) fields from 5 sections were scored. $n = 5$. Angiogenic scores were calculated as percentage increases in PECAM-1 staining in Ad-VEGF versus Ad–β-gal–treated ears. $^{*}P < 0.05$; $^{**}P < 0.01$.

residues examined is via Akt1. Interestingly, MDM2 phosphorylation increased with Akt1 deficiency and decreased with reintroduction of Akt1, which suggests that Akt1 may negatively regulate an additional kinase to phosphorylate S166.

Since Akt is important for cell migration in many cell types, we examined the migration of MLECs and MLFs in a modified Boyden chamber assay. Sphingosine-1-phosphate is a potent chemoattractant for ECs that utilizes a PI3K/Akt pathway (5, 6). As shown in Figure 6A, sphingosine-1-phosphate–induced migration was reduced in *Akt1*$^{-/-}$ MLECs and in *Akt1*$^{-/-}$ MLECs reconstituted with GFP (*Akt1*$^{-/-}$rcGFP), and the reintroduction of Akt1 in *Akt1*$^{-/-}$ MLECs rescued the defective migratory phenotype (*Akt1*$^{-/-}$rcAkt1). Next we measured serum-induced migration in MLFs. As shown in Figure 6B, the basal migration was similar in WT, *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$rcGFP cells whereas *Akt1*$^{-/-}$rcAkt1 cells exhibited higher basal migration consistent with overexpression of Akt promoting chemokinesis (17). However, consistent with data in MLECs, the loss of Akt1, but not Akt2, reduced serum-induced migration of fibroblasts, an effect rescued by the reintroduction of Akt1.

eNOS is an Akt substrate in ECs that produces the second messenger gas, NO. Phosphorylation of eNOS on S1176 in mice (equivalent to S1179 and S1177 for bovine and human, respectively) by Akt increases the rate of electron flux through NOS, changes its calcium sensitivity, and augments NO release (18, 20, 21). NO, in turn, can regulate blood flow and aspects of angiogenesis, including cell migration, cell proliferation, and tube formation (22). Interestingly, mice deficient in eNOS exhibit defects in ischemia, VEGF-induced angiogenesis, vascular permeability, and EPC mobilization akin to that seen in *Akt1*$^{-/-}$ mice (23–26). To determine whether the loss of Akt1 influences eNOS activation, we measured NO release from ECs isolated from WT, Akt1, and *Akt2*$^{-/-}$ mice. As shown in Figure 6C, the basal accumulation of NO (over 24 hours) is similar in MLECs cultured from WT, *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$ ECs reconstituted with Akt1; however, the loss of Akt1 markedly reduced NO accumulation (Figure 6C). Similar trends were seen in ECs acutely stimulated with the angiogenic cytokine VEGF (Figure 6D). VEGF promoted the release of NO from WT, *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$ ECs reconstituted with Akt1; however, the loss of Akt1 markedly reduced VEGF-stimulated NO accumulation. In contrast, ionomycin-stimulated NO release was not different among the groups (ionophore-stimulated NO release was 1.29 ± 0.51, 1.36 ± 0.01, and 1.51 ± 0.13 nmol/mg protein in WT, *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ ECs, respectively; $n = 3$ repeated twice). These data are consistent with the diminution of VEGF coupling to eNOS by inhibition of PI3K, dominant-negative Akt, or p-defective mutants of eNOS lacking the serine phosphorylation site (3, 17, 18, 22). Therefore, the postnatal angiogenic defects in *Akt1*$^{-/-}$ mice, i.e., the loss of ischemia and VEGF-induced angiogenesis as well as a reduction in EPC mobilization and function in response to tissue ischemia, can be explained in part by defects in Akt substrate phosphorylation, cell migration, and NO release, all mechanisms important for the assembly of stable angiogenic vessels.

Discussion

Our study provides in vivo genetic data supporting a role for Akt1 in regulating ischemic and VEGF-initiated postnatal angiogenesis and has broad implications for understanding the physiology and importance of Akt in vivo. First, the lack of arteriogenesis and angiogenesis secondary to ischemia and the blunted response to VEGF in mice deficient in Akt1 clearly indicates that the genes critically involved in postnatal angiogenesis are different from those important during vascular development or parturition. For example, the genetic loss of VEGF and its receptors results in early embryonic lethality (27–30) whereas the loss of eNOS, Akt1, or placenta-derived growth factor does not influence vascular development. However, in response to tissue injury or exogenous angiogenic factors, the loss of eNOS (23, 25), Akt (this study), and placenta-derived growth factor (31) results in severe peripheral vascular disease and, in the case of eNOS and Akt, limb loss. Thus, permissive signaling pathways not important for vascular development may exert profound actions postnatally after stressful challenge

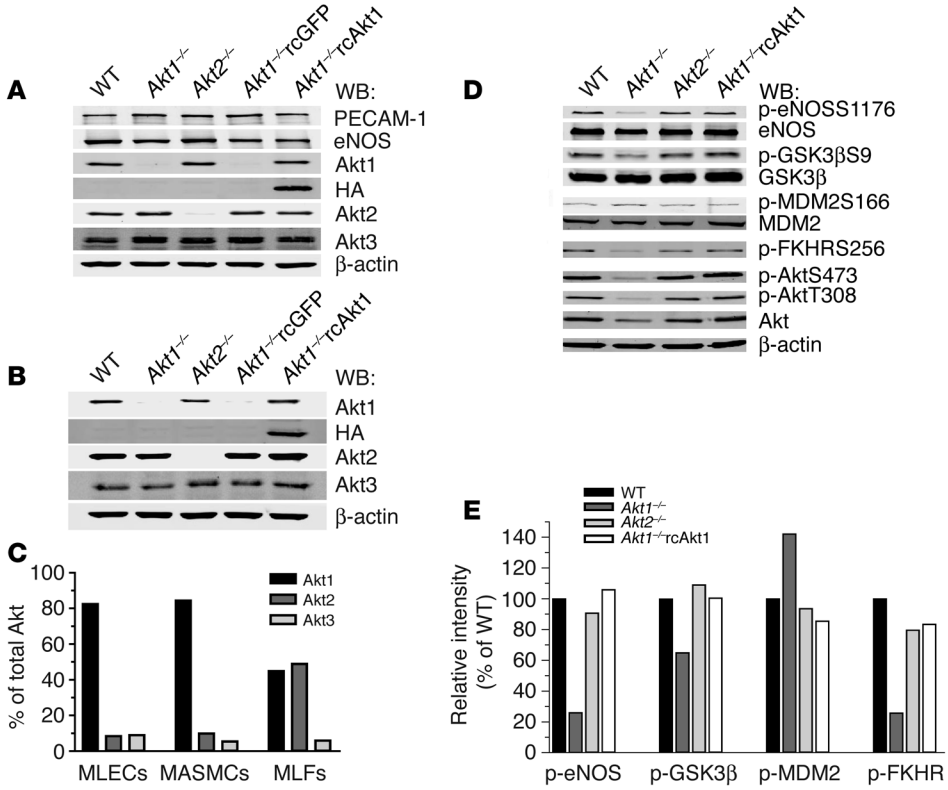

Figure 5
Characterization of Akt-deficient ECs and fibroblasts. (**A**) Characterization of MLECs. Lung ECs were isolated from *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, and WT littermates. Some *Akt1*$^{-/-}$ ECs were reconstituted with a retrovirus expressing HA-tagged Akt1 (*Akt1*$^{-/-}$rcAkt1) or with GFP (*Akt1*$^{-/-}$rcGFP). WB, Western blot. (**B**) Characterization of mouse lung fibroblasts. (**C**) Relative expression of Akt isoforms in mouse cells. Lysates from MLECs, MLFs, and MASMCs were analyzed by SDS-PAGE and quantitative Western blot. Relative protein amounts of Akt1, Akt2, and Akt3 in the cells were quantified using standard curves obtained by running recombinant mouse Akt1, Akt2, and Akt3 proteins. (**D**) Basal phosphorylation of Akt and Akt substrates in MLECs. Cells were serum-starved for 48 hours and cell lysates subjected to SDS-PAGE and Western blot using the indicated antibodies. (**E**) Densitometric quantitation of p-protein to total protein for eNOS, GSK3β, and MDM2. For p-FKHR, data is expressed relative to β-actin loading control.

or exogenous angiogenic factor administration. Secondly, our data imply that although Akt1, Akt2, and, to a lesser extent, Akt3, were expressed in the vascular cells examined, Akt1 exerts a nonredundant effect in modulating substrate phosphorylation, angiogenesis, EPC mobilization, cell migration, and cell coupling to eNOS. These data are consistent with earlier studies documenting disordered vasculature and impaired eNOS phosphorylation in placentas of *Akt1*$^{-/-}$ mice (11). The high abundance of Akt2 in the vessel walls, likely reflecting the fibroblast population of the adventitia (based on the expression of Akt2 in isolated fibroblasts), suggests that Akt2 may be involved in other functions related to the adventitia such as injury-evoked vascular remodeling.

The defects in ischemia and VEGF-mediated angiogenesis exhibited in *Akt1*$^{-/-}$ mice are indeed multifaceted. Secondary to tissue ischemia, macrophage-derived cytokines, changes in shear stress, and metalloproteinase activity are thought to initiate arteriogenesis and lower-limb angiogenesis (32, 33). In addition to local regulation of arteriogenesis and angiogenesis, there is increasing evidence that paracrine regulation of these processes may occur via EPCs. Indeed, ischemia or VEGF induces the mobilization of EPCs into the circulation that may contribute to ischemic arteriogenesis and angiogenesis (34, 35); however, this view is not accepted by all (36, 37). The inefficient mobilization of EPCs from *Akt1*$^{-/-}$ mice and the lack of improvement in blood flow recovery after injection of *Akt1*$^{-/-}$ EPCs is consistent with our cell culture data showing a critical role for Akt1 in mediating chemotaxis and NO release. This is consistent with impaired ischemic responses, EPC mobilization, and EPC function in eNOS knockout mice (25). However, in the present study we have not explored the ability of *Akt1*$^{-/-}$ cells to integrate into existing vasculature, fuse, or release cytokines implicated in arteriogenesis/angiogenesis. In the context of cell migration, our data clearly show that Akt1 is necessary for mammalian cell migration (both ECs and fibroblasts), thus providing genetic evidence for the similar requirement of Akt for polarized cell migration in *Dictyostelium discoideum* (38). The role of Akt in EC migration is supported by previous studies showing that inhibition of PI3K, expression of dominant-negative Akt, and blockage of eNOS all reduce chemokinesis (17, 39), which confirms that a PI3K/Akt/eNOS module is important for coupling to chemotactic signaling in ECs.

Another salient feature of this study is that we provide the first direct in vivo evidence to our knowledge that VEGF-induced changes in vascular leakage require Akt1, supporting previous work using dominant-negative and constitutively active Akt (40). Previous genetic data have shown that cellular src (c-src; ref. 41) and eNOS (24, 26) are both downstream mediators of VEGF-induced changes in vascular permeability. Since c-src may lie upstream of Akt activation (42) and upstream or downstream of eNOS activation (42–44), it is feasible that blockage of c-src reduces VEGF-induced vascular permeability by reducing Akt phosphorylation and eNOS activation. Finally, our data suggest that inhibition of Akt1 signaling may be an effective strategy to block not only tumor growth but also pathological angiogenesis mediated by VEGF. However, this strategy may also worsen peripheral vascular disease secondary to atherosclerosis, diabetes, or aging.

Methods

Animals. *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice were generated as previously described (8, 9). F2 and F3 generation, male, 8- to 14-week-old knockout mice and their WT littermates were used. All experiments were approved by the Institutional Animal Care and Use Committee of Yale University.

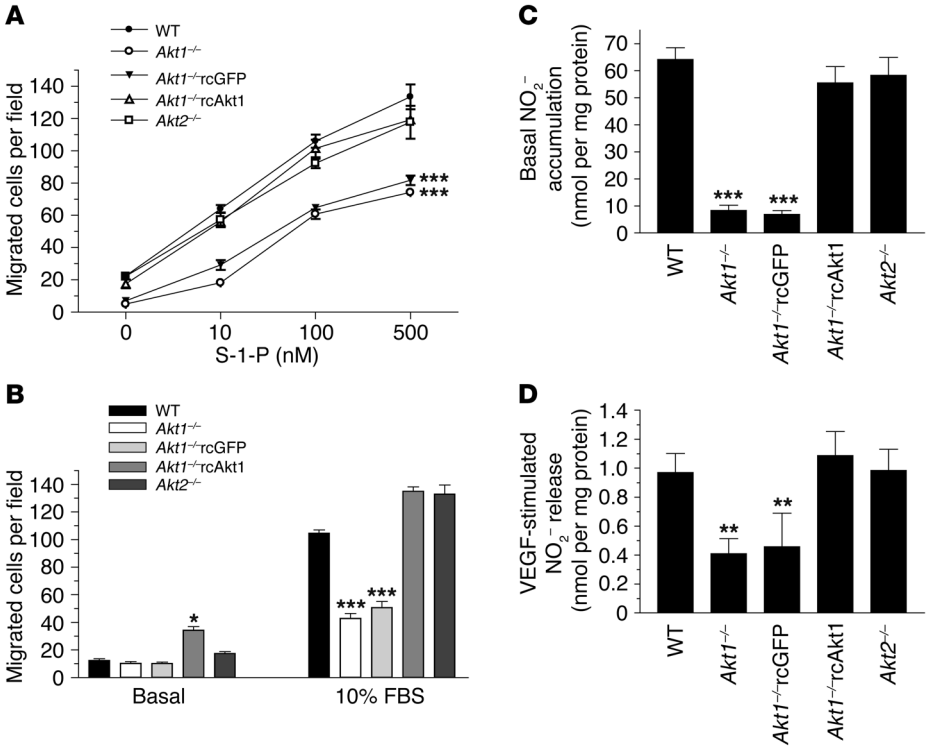

Figure 6
Impaired cell migration and NO release in *Akt1*$^{-/-}$ cells. (**A**) Migration of MLECs was examined in modified Boyden chambers using sphingosine-1-phosphate (S-1-P) as a chemoattractant. Migration of *Akt1*$^{-/-}$ cells was reduced at all doses examined. Data are mean ± SEM; $n = 4$ from 3 independent experiments. (**B**) Migration of lung fibroblasts was examined in the modified Boyden chamber using serum-free DMEM (Basal) or 10% FBS as agonist. (**C**) Basal production of NO in MLECs (assayed as NO_2^- in the media) over a 24-hour period was determined by chemiluminescence. Levels of NO_2^- in media alone were subtracted out. (**D**) Cells were stimulated with VEGF (50 ng/ml) for 30 minutes, and NO release was quantified by chemiluminescence. For stimulated NO_2^- release, values were calculated by subtracting out levels obtained with nonstimulated cells. Data are mean ± SEM; $n = 9$–12 from 3 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Hindlimb ischemia model. Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The left femoral artery was ligated at the point of entry through the inguinal ligament, at the origin of the popliteal artery, and at midway through the saphenous artery. Small branches were cauterized, and the portion of the artery between the ligatures was removed. Blood flow was measured using a deep penetrating laser Doppler probe (Perimed) placed directly on the gastrocnemius muscle. Flow measurements were made just before, immediately after, and at 2 and 4 weeks after femoral arteriectomy. At 4 weeks after femoral resection, gastrocnemius and soleus muscles from the ischemic and control limbs were fixed with methanol and embedded in paraffin. Five-micron sections were stained with TRITC-labeled lectin (Bandeiraea simplicifolia 1; Sigma-Aldrich).

Ear angiogenesis. Adenoviruses encoding murine VEGF-A164 (7×10^8 viral particles) were injected intradermally into the left ears of *Akt1*$^{-/-}$ or WT littermate mice. The right ears were injected with a control virus expressing β-gal. After 5 days, animals were euthanized and the ears removed and embedded in OCT compound (Tissue-Tek; Sakura). Frozen sections (7 μm thick) were immunostained with monoclonal anti-mouse PECAM-1 primary (BD Biosciences — Pharmingen) and Alexa 488–conjugated secondary antibodies. PECAM-1–positive staining was quantified using Openlab software (version 4.0.2; Improvision).

Modified Miles assay. Under anesthesia, mice were injected with Evans blue (30 mg/kg; Sigma-Aldrich). VEGF (300 ng in 15 μl) or saline was injected subcutaneously into the dorsal surface of the right and left ears, respectively. After 30 minutes, mice were euthanized and the ears were removed, oven-dried at 55°C, and weighed. Evans blue was then extracted from the ears using 500 μl of formamide for 24 hours at 55°C. Evans blue extravasation into the ear was measured spectrophotometrically at 630 nm using a standard curve of Evans blue in formamide.

Isolation of mononuclear cells and EPC culture. Mice were anesthetized with Avertin (2.5%), and blood was drawn by cardiac puncture. Mononuclear cells were isolated by a density gradient method using Histopaque-1077 (Sigma-Aldrich). Heparinized blood (500 μl) was mixed with 2 ml PBS, gently added to 2 ml Histopaque-1077, and centrifuged at 400 *g* for 30 minutes. The mononuclear fraction was collected and washed in PBS; following red cell lysis with ammonium chloride solution (StemCell Technologies), 1×10^6 cells/cm^2 were seeded on fibronectin-coated slides (Biocoat). Cells were allowed to differentiate in endothelial growth medium–2 (EGM-2) SingleQuots medium (Clonetics) containing VEGF-A, FGF, IGF-1, EGF, hydrocortisone, ascorbic acid, gentamicin and amphotericin-B, heparin, 1% gentamicin/streptomycin (Invitrogen Corp.), and 5% FBS. Medium was changed every other day. After 5 days of in vitro culture, cells were incubated in 10 μg/ml dioctadecyl-3,3,3′,3′-tetrametylindocarbocyanine perchlorate–labeled acetylated LDL (Ac-Dil-LDL; Biomedical Technologies Inc.) for 5 hours, and Ac-Dil-LDL–positive cells were photographed at 560 nm on a Nikon Eclipse Fluorescence microscope (Nikon). Ac-Dil-LDL–positive cells in 10 low-power fields were counted for each animal.

In the EPC reconstitution experiments, EPCs were isolated from splenic cell homogenates as previously described (25). The mononuclear cells isolated by density centrifugation were plated on fibronectin-coated plates and incubated in the above medium for 4 days. EPCs were collected by trypsin treatment and dissolved in EGM-2 media. WT mice underwent hindlimb ischemia as described above. On the day of surgery, WT mice received an intravenous infusion of 2×10^5 EPCs isolated from WT or Akt1 knockout mice. Control mice were injected with growth factor–free EGM-2 media alone. Revascularization was assessed by laser Doppler blood-flow analysis at 14 days after surgery as previously described (45).

MLEC isolation. MLECs were isolated from 3-week-old *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, and WT mice. Briefly, mice were euthanized with an overdose of ketamine/xylazine and the lungs were excised, minced, and digested with 0.1% collagenase in RPMI medium. The digest was homogenized by passing multiple times through a 14-gauge needle. It was then filtered through a 150-μm tissue sieve, and the cell suspension was plated on 0.1 % gelatin-coated dishes.

After 2 to 3 days, cells were immortalized by 2 rounds of infection with retrovirus encoding the middle T antigen. Cells were allowed to recover for 24 hours, and then ECs were isolated by immunoselection with PECAM-1– and ICAM-2–conjugated magnetic beads. When cells reached confluency, a second round of immunoisolation was performed. Cells were propagated in EGM-2 media supplemented with EGM-2 microvascular (MV) SingleQuots (Cambrex). For *Akt1*$^{-/-}$ reconstitution, cells were infected twice with a retrovirus encoding either HA-tagged murine Akt1 or GFP.

Cell migration assay. Cell migration assays were performed using a modified Boyden chamber with Costar Transwell inserts (Corning). The inserts were coated with a solution of 0.1% gelatin. Subconfluent MLECs or MLFs were serum-starved overnight. Solutions of sphingosine-1-phosphate (10–500 nm) or 10% FBS were prepared in DMEM containing 0.1% BSA and added to the bottom chambers. MLECs or MLFs (2×10^5 cells) were added to the upper chambers. After 4 hours incubation at 37°C, cells on both sides of the membrane were fixed and stained with the DiffQuik staining kit (Baxter). Cells on the upper side of the membrane were removed with a cotton swab. The average number of cells per field on the lower side of the membrane from 4 high-power (×400) fields was counted.

NO release from cells. MLECs (passage 4 to 10) were used for NO release experiments. Basal and VEGF-stimulated NO_2^- release were assessed in the medium as previously described (18).

Western blot analysis. Tissues were snap frozen in liquid nitrogen, pulverized, and resuspended in lysis buffer: 50 mM Tris-Hcl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM Pefabloc SC, and 2 mg/ml protease inhibitor cocktail (Roche Diagnostics Corp.). Cells were lysed on ice with lysis buffer as noted above. Protein concentrations were determined using the D_C Protein assay kit (Bio-Rad Laboratories). Lysates containing 80 μg (tissue) or 30 μg (cells) of protein were analyzed by SDS-PAGE and immunoblotting. Primary antibodies used include the following: Akt1 mAb (Upstate), Akt2 p-Ab (46), eNOS mAb, heat shock protein 90 (Hsp90), mAb (BD Transduction Laboratories; BD Biosciences — Pharmingen), anti-HA mAb (Roche Diagnostics Corp.), PECAM-1 p-Ab (Santa Cruz Biotechnology Inc.), β-actin mAb (Sigma-Aldrich), and p-eNOS S1179 p-Ab (Zymed Laboratories Inc.). Secondary antibodies were fluorescent-labeled antibodies (LI-COR Biotechnology). Bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Quantitative Western blots. For quantification of Akt isoform expression, recombinant Akt1, Akt2, and Akt3 proteins were purchased from Upstate and the concentration of each recombinant protein determined by analysis of a Coomassie-stained gel using the Odyssey system (LI-COR Biotechnology) and a bovine serum albumin standard. A standard curve of each recombinant Akt protein and dilutions of cell lysate were resolved on 10% SDS-PAGE, transferred to nitrocellulose, and probed with isoform-specific antibodies (Akt1: Upstate; Akt2: ref. 46; Akt3: ref. 47). Secondary antibodies were labeled with IRDye 800 (Rockland Immunochemicals) for quantification by the Odyssey system. The total Akt protein was determined as the sum of the individual isoforms; the amount of each isoform is expressed as a percentage of the total Akt protein.

Statistics. Data are expressed as mean ± SEM. Comparisons between groups were made using a 2-tailed Student's *t* test or ANOVA with the Bonferroni post hoc test. Differences were considered to be significant at $P < 0.05$.

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1. Mazure, N.M., Chen, E.Y., Laderoute, K.R., and Giaccia, A.J. 1997. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood.* **90**:3322–3331.
2. Gerber, H.P., et al. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* **273**:30336–30343.
3. Fujio, Y., and Walsh, K. 1999. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J. Biol. Chem.* **274**:16349–16354.
4. Papapetropoulos, A., et al. 2000. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J. Biol. Chem.* **275**:9102–9105.
5. Lee, M.J., et al. 2001. Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol. Cell.* **8**:693–704.
6. Morales-Ruiz, M., et al. 2001. Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J. Biol. Chem.* **276**:19672–19677.
7. Kureishi, Y., et al. 2000. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat. Med.* **6**:1004–1010.
8. Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F., and Birnbaum, M.J. 2001. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J. Biol. Chem.* **276**:38349–38352.
9. Cho, H., et al. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science.* **292**:1728–1731.
10. Chen, W.S., et al. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev.* **15**:2203–2208.
11. Yang, Z.Z., et al. 2003. Protein kinase B alpha/Akt1 regulates placental development and fetal growth. *J. Biol. Chem.* **278**:32124–32131.
12. Asahara, T., et al. 1999. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18**:3964–3972.
13. Rehman, J., Li, J., Orschell, C.M., and March, K.L. 2003. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation.* **107**:1164–1169.
14. Badorff, C., et al. 2003. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation.* **107**:1024–1032.
15. Chan, J., Bayliss, P.E., Wood, J.M., and Roberts, T.M. 2002. Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell.* **1**:257–267.
16. Jiang, B.H., Zheng, J.Z., Aoki, M., and Vogt, P.K. 2000. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **97**:1749–1753.
17. Morales-Ruiz, M., et al. 2000. Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ. Res.* **86**:892–896.
18. Fulton, D., et al. 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* **399**:597–601.
19. Summers, S.A., Lipfert, L., and Birnbaum, M.J. 1998. Polyoma middle T antigen activates the Ser/Thr kinase Akt in a PI3-kinase-dependent manner. *Biochem. Biophys. Res. Commun.* **246**:76–81.
20. Dimmeler, S., et al. 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* **399**:601–605.
21. Michell, B.J., et al. 1999. The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr. Biol.* **9**:845–848.
22. Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A., and Sessa, W.C. 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100**:3131–3139.
23. Murohara, T., et al. 1998. Nitric oxide synthase

modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101**:2567–2578.
24. Fukumura, D., et al. 2001. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc. Natl. Acad. Sci. U. S. A.* **98**:2604–2609.
25. Aicher, A., et al. 2003. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat. Med.* **9**:1370–1376.
26. Gratton, J.P., et al. 2003. Selective inhibition of tumor microvascular permeability by cavtratin blocks tumor progression in mice. *Cancer Cell.* **4**:31–39.
27. Fong, G.H., Rossant, J., Gertsenstein, M., and Breitman, M.L. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* **376**:66–70.
28. Shalaby, F., et al. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature.* **376**:62–66.
29. Carmeliet, P., et al. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* **380**:435–439.
30. Ferrara, N., et al. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* **380**:439–442.
31. Carmeliet, P., et al. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* **7**:575–583.
32. Heil, M., and Schaper, W. 2004. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ. Res.* **95**:449–458.
33. Scholz, D., et al. 2002. Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J. Mol. Cell. Cardiol.* **34**:775–787.
34. Takahashi, T., et al. 1999. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.* **5**:434–438.
35. Yamamoto, K., et al. 2004. Molecular evaluation of endothelial progenitor cells in patients with ischemic limbs: therapeutic effect by stem cell transplantation. *Arterioscler. Thromb. Vasc. Biol.* **24**:e192–e196.
36. Ziegelhoeffer, T., et al. 2004. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ. Res.* **94**:230–238.
37. Khmelewski, E., Becker, A., Meinertz, T., and Ito, W.D. 2004. Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ. Res.* **95**:E56–E64.
38. Chung, C.Y., Potikyan, G., and Firtel, R.A. 2001. Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. *Mol. Cell.* **7**:937–947.
39. Dimmeler, S., Dernbach, E., and Zeiher, A.M. 2000. Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett.* **477**:258–262.
40. Six, I., Kureishi, Y., Luo, Z., and Walsh, K. 2002. Akt signaling mediates VEGF/VPF vascular permeability in vivo. *FEBS Lett.* **532**:67–69.
41. Eliceiri, B.P., et al. 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol. Cell.* **4**:915–924.
42. Haynes, M.P., et al. 2003. Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen. *J. Biol. Chem.* **278**:2118–2123.
43. Davis, M.E., Cai, H., Drummond, G.R., and Harrison, D.G. 2001. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circ. Res.* **89**:1073–1080.
44. Davis, M.E., Cai, H., McCann, L., Fukai, T., and Harrison, D.G. 2003. Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training. *Am. J. Physiol. Heart Circ. Physiol.* **284**:H1449–H1453.
45. Shibata, R., et al. 2004. Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of AMP-activated protein kinase signaling. *J. Biol. Chem.* **279**:28670–28674.
46. Calera, M.R., et al. 1998. Insulin increases the association of Akt-2 with Glut4-containing vesicles. *J. Biol. Chem.* **273**:7201–7204.
47. Easton, R.M., et al. 2005. Role for Akt/protein kinase Bgamma in attainment of normal brain size. *Mol. Cell. Biol.* **25**:1869–1878.