Endothelial Grb2-Associated Binder 1 Is Crucial for Postnatal Angiogenesis

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Objective—Grb2-associated binder 1 (Gab1), a scaffolding adaptor protein, plays an important role in transmitting key signals that control cell growth, differentiation, and function from multiple tyrosine kinase receptors. The study was designed to investigate the role of endothelial Gab1 in angiogenesis and its underlying molecular mechanisms.

Methods and Results—Using cre-loxp technology, we generated endothelial-specific Gab1 knockout (Gab1-ecKO) mice. Gab1-ecKO mice are viable and showed no obvious developmental defects in the vascular system. To analyze the role of Gab1 in postnatal angiogenesis, we used hindlimb ischemia and Matrigel plug models. We found that loss of endothelial Gab1 in mice dramatically impaired postnatal angiogenesis. Gab1-ecKO mice had impaired ischemia-initiated blood flow recovery, exhibited reduced angiogenesis, and were associated with marked limb necrosis. We further observed significant endothelial cell (EC) death in the ischemic hindlimb of Gab1-ecKO mice. Matrigel plug assay showed that hepatocyte growth factor (HGF)–mediated angiogenesis was inhibited in Gab1-ecKO mice. In vitro studies showed that Gab1 was required for HGF-induced EC migration, tube formation, and microvessel sprouting. Mechanistically, HGF stimulated Gab1 tyrosine phosphorylation in ECs, leading to activation of ERK1/2 and Akt, which are angiogenic and survival signaling.

Conclusion—Gab1 is essential for postnatal angiogenesis through mediating angiogenic and survival signaling. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ■ endothelium ■ growth factors ■ ischemia ■ Gab1

Angiogenesis, the formation of new blood vessels from the existing vascular network, begins with the activation, migration, and proliferation of endothelial cells (ECs) in the existing vessels. Many growth factors, including vascular endothelial growth factor (VEGF), are shown to regulate EC function and angiogenesis. Besides VEGF, hepatocyte growth factor (HGF) is a potent angiogenic factor in vivo and stimulates vascular EC migration, proliferation, and organization into capillary-like tubes in vitro. HGF binds its receptor c-Met and stimulates c-Met kinase activation, which triggers transphosphorylation of c-Met and downstream signaling events. The HGF/c-Met pathway has emerged as a promising therapeutic target by which to promote or inhibit angiogenesis. However, the mechanisms by which HGF mediates angiogenesis have not been fully understood.

Grb2-associated binder 1 (Gab1), a member of the insulin receptor substrates 1–like multi-substrate docking protein family, is highly expressed in vascular ECs. Gab1 is found to be directly or indirectly recruited to numerous activated receptor tyrosine kinases, including c-Met and VEGF receptor 2 (also known as Flk1 and KDR). On phosphorylation by receptor tyrosine kinase, Gab1 then recruits and activates phosphatidylinositol 3-kinase (PI3K)/Akt and protein tyrosine phosphatase SHP2/ERK1/2 pathways, which are crucial to cell proliferation and differentiation. However, the role of Gab1 in growth factors/receptor tyrosine kinase–mediated angiogenesis remains largely unclear.

Gab1 deficiency in mice is early embryonic lethal because of heart and placental defects. To directly explore the role of Gab1 in angiogenesis, we generated endothelial-specific Gab1 knockout (Gab1-ecKO) mice. Using hindlimb ischemia, Matrigel plug, and tumor angiogenesis models, we showed defective postnatal angiogenesis in Gab1-ecKO mice. We further found that Gab1 was required for EC survival in vivo and in vitro under stress conditions. Moreover, HGF-induced Akt and ERK1/2 activation and cell survival were impaired in Gab1-deficient ECs. Thus, our data demonstrate that Gab1 is crucial for postnatal angiogenesis through mediating angiogenic and survival signaling.

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Methods

Animals

Gab1^flx/flx^ mice (C57BL/6J background) described previously and Tie2-Cre transgenic mice from the Jackson Laboratory (C57BL/6J background, stock number 008863) were used. All protocols for animal experiments were approved by the University Committee on Animal Resource of University of Rochester.

Hindlimb Ischemia Model

Hindlimb ischemia was generated by resection of the femoral artery in male wild-type (WT) or Gab1-ecKO mice as previously described. Laser Doppler perfusion imaging was used to record perfusion of both right and left limbs at different time points as indicated.

An expanded methods section is available in the Supplemental Data (available online at http://atvb.ahajournals.org), including hindlimb ischemia model, laser Doppler perfusion imaging, capillary density analysis, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, in vivo Matrigel plug angiogenesis assay, tumor angiogenesis, aortic ring assay for ex vivo angiogenesis, isolation of mouse lung ECs, cell culture and small interfering RNA (siRNA) transfection, wound closure cell migration, Boyden chamber migration, capillary-like tube formation, Western blot analysis, immunofluorescent staining and microscopy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cell viability assay, and data statistical analysis.

Results

Generation and Characterization of Gab1-ecKO Mice

To explore the role of endothelial Gab1 in vivo, we generated Gab1 endothelium-specific knockout mice. Gab1^flx/flx^ mice were bred to Tie2-Cre/+ transgenic mice that express Cre in ECs. Tie2-Cre;Gab1^flx/flx^ (Gab1-ecKO) mice were generated by male Tie2-Cre/Gab1^flx/flx^ and female Gab1^flx/flx^ intercross. Gab1-ecKO mice were viable and were born at the expected mendelian ratio (Supplemental Table I). Supplemental Figure I shows the genotyping results for Gab1-ecKO mice and Gab1^flx/flx^ WT (control) littermates. Gab1-ecKO mice had no obvious defects on body weight and vascular development. Lung ECs were isolated. EC morphology was normal (Supplemental Figure II) and confirmed with endothelial specific markersPECAM1 (CD31) and VE-cadherin immunostaining (Supplemental Figure III). Immunoblotting analysis showed a significant decrease of Gab1 expression in ECs isolated from Gab1-ecKO mice relative to those from WT mice (Supplemental Figure IV). Coinmunostaining Gab1 with the EC-specific marker von Willebrand factor (vWF) in mouse hindlimb muscles further confirmed EC-specific Gab1 deficiency in Gab1-ecKO mice (Supplemental Figure V), whereas a relatively low expression of Gab1 in muscle fibers in both Gab1-ecKO mice and WT mice was observed. Moreover, we found that the hindlimb vasculature and retina vascular network in adult Gab1-ecKO mice were similar to those in WT mice (Supplemental Figures VI and VII). Collectively, our results indicate that Gab1-ecKO mice have no obvious developmental defects in the vascular system.

Gab1 Is Essential for Ischemia-Initiated Blood Flow Recovery and Angiogenesis

To examine the role of endothelial Gab1 in postnatal angiogenesis, we used a mouse hindlimb ischemia model. The gastrocnemius blood flow was measured using a deep penetrating laser Doppler probe. As shown in Figure 1A and 1B, the ratio of blood flow of the right limb relative to the left limb was 100% before surgery. After surgery to the right limb, the ratios dropped by 80% in all groups of WT and Gab1-ecKO mice. Blood flow in the gastrocnemius muscle in WT mice recovered in a time-dependent manner. However, Gab1-ecKO mice failed to recover the blood flow, suggesting that Gab1 may play a role in postnatal ischemic angiogenesis. Moreover, Gab1-ecKO mice showed marked necrotic limb at 14 days after the femoral artery resection (Figure 1C), which is consistent with the defective recovery of blood flow. We used a clinical scoring system to assess lower-limb function and tissue salvage after surgery. As shown in Figure 1D, the deficiency of endothelial Gab1 was associated with severe tissue ischemia after surgery.

To characterize the ischemia-initiated angiogenesis, we quantified the capillary density of gastrocnemius muscle fibers from both WT and Gab1-ecKO mice before and after the hindlimb ischemia. As shown in Figure 1E, ischemia-initiated angiogenesis resulted in increased capillary density relative to the baseline in WT mice, whereas Gab1-ecKO mice had dramatically lower capillary density, less than that in the nonischemic control side (Supplemental Figure VIII shows color images of capillary CD31 staining). Taken together, these results established a critical role of endothelial Gab1 in ischemia-initiated angiogenesis.

Gab1 Is Critical for EC Survival Under Stress Conditions

Notably, Figure 1E, showing a dramatically decreased capillary density in Gab1-ecKO mouse, suggests the potential involvement of EC survival in vivo under hypoxia, which results in defective angiogenesis and vascular regression. To directly examine the role of Gab1 in cell survival, we performed TUNEL staining on tissues from Gab1-ecKO mice and WT mice after hindlimb ischemic surgery. Endothelial marker gene vWF and nDNA 4′,6-diamidino-2-phenylindole staining were also performed to identify ECs and cell nuclei. As shown in Figure 2, there were significantly more TUNEL-positive ECs in gastrocnemius muscle in Gab1-ecKO mice than in those of WT mice after ischemia. EC apoptosis in the arterioles was further confirmed by triple immunostaining with TUNEL, vWF, and smooth muscle α-actin (Supplemental Figure IX). It is worth noting that EC death started as early as 2 days (Figure 2A and 2B) and persisted at 7 days (Figure 2C and 2D) after ischemia in Gab1-ecKO mice, revealing a pivotal role of Gab1 in EC survival under ischemic stress.

It has been reported that Gab1 is involved in VEGF-induced EC survival. HGF is also a potent survival growth factor. We observed that expression of both VEGF and HGF was increased in ischemic hindlimb muscles (Supplemental Figure X). Interestingly, we found that HGF strongly induced Gab1 tyrosine phosphorylation in ECs (Supplemental Figure XI). To examine whether Gab1 mediates HGF-induced EC survival under stress conditions, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium cell viability assay and mouse lung ECs isolated from Gab1-ecKO mice and WT mice. Under serum starvation for 24 hours, cell viability of Gab1-deficient ECs dropped by almost 55% relative to the control WT ECs (Figure 2E). Although HGF significantly protected WT ECs from death,
it failed to maintain EC survival in Gab1-deficient ECs (Figure 2E). Less extensive effects were observed when VEGF was used to protect ECs from death (Supplemental Figure XII). We also examined the levels of cleaved caspase 3 for the induction of apoptosis and found that increased active caspase 3 in response to serum starvation was much higher in Gab1-deficient ECs than in WT ECs (Supplemental Figure XIII). These data show that Gab1 is required for EC survival under serum starvation.

Gab1 Is Necessary for HGF-Induced Angiogenesis Revealed by Matrigel Plug Assay

To dissect the role of Gab1 in HGF-induced angiogenesis in vivo, we used the Matrigel plug model, in which Matrigel mixed with HGF was subcutaneously injected into Gab1-ecKO mice and WT mice. Matrigel plugs were harvested 1 week later and stained for CD31. As shown in Figure 3A, deficiency of endothelial Gab1 in mice significantly reduced invading ECs and the density of microvessels in the Matrigel plugs (Supplemental Figure XIV shows color images of capillary CD31 staining). Hemoglobin concentration in the Matrigels was measured to assess the functional vessels in the Matrigel plugs. As shown in Figure 3B, hemoglobin concentration was much lower in Matrigel plugs implanted in Gab1-ecKO mice than in those in Gab1 WT mice, consistent with the results of CD31 staining for microvessels in Matrigel plugs. These data demonstrate that Gab1 is essential for HGF-induced angiogenesis in vivo. Relatively lower effects of endothelial Gab1 deficiency on VEGF-induced angiogenesis were observed by the Matrigel plug assay using Gab1-ecKO mice and WT mice (Supplemental Figure XV). In addition, we examined the role of Gab1 in tumor angiogenesis and found that endothelial Gab1 deficiency inhibited tumor angiogenesis and tumor growth (see Supplemental Figures XVI and XVII).

Gab1 Mediates Microvessel Sprouting, Capillary-Like Tube Formation, and EC Migration

To further investigate the role of Gab1 in angiogenesis, we performed the aorta ring ex vivo angiogenesis assay, in vitro angiogenesis capillary-like tube formation, and EC migration. In the aorta ring assay, we found that the number of sprouting microvessels from the aorta rings isolated from Gab1-ecKO mice in the presence of HGF was dramatically reduced (Figure 4A and 4B). In the capillary-like tube formation assay, we used mouse lung ECs isolated from Gab1-ecKO mice and WT mice. As shown in Figure 4C and 4D, the capillary-like tube formation in the presence of HGF was dramatically reduced. Cell migration was measured by the modified Boyden chamber method and wound healing migration assay. In the modified Boyden chamber method, HGF-induced EC migration was impaired in Gab1-deficient ECs compared with WT ECs (Figure 4E). Lower effects of endothelial Gab1 deficiency on VEGF-induced EC migration were observed.

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**Figure 1.** Gab1-ecKO mice have impaired ischemia-initiated blood flow recovery and angiogenesis. A, Representative images of laser Doppler blood flow before, immediately after, and on days 1, 3, 7, and 14 after femoral artery resection. B, Blood flow in ischemic hind limb was measured. Results are expressed as a ratio of the right (ischemic) to left (control, nonischemic) limb perfusion. WT (n=13), and Gab1-ecKO (n=10). *P<0.05 vs WT. C, Gab1-ecKO mice developed necrotic toes at 7 to 14 days after femoral artery resection, whereas WT littermate mice did not. Representative images for necrotic toes (arrow) vs WT control at 14 days after surgery are shown. D, Clinical score at 14 days after femoral arteriotomy 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=13; Gab1-ecKO, n=10. *P<0.05 vs WT. E, Quantification of capillary density, calculated as the number of capillaries per muscle fiber. For each animal, 6 or 7 randomly selected fields from 3 to 5 sections were counted. WT, n=13; Gab1-ecKO, n=10. *P<0.05 vs WT control, **P<0.05 vs WT ischemic.
Gab1 Is Required for Angiogenic and Survival Signaling in ECs

The PI3K/Akt and ERK signaling pathways are involved in EC survival and in vitro angiogenesis. To investigate how Gab1 mediates HGF-induced EC survival and migration, we assessed the activation of Akt and ERK1/2 in response to HGF stimulation using human umbilical vein endothelial cells (HUVECs) transfected with Gab1 siRNA or scrambled siRNA (control). Gab1 siRNA efficiently reduced the level of Gab1 expression in HUVECs (Figure 5A). Knockdown Gab1 substantially decreased the levels of Akt and ERK1/2 phosphorylation in response to HGF (Figure 5A to 5C). We also used Gab1-deficient ECs to examine HGF-induced Akt and ERK1/2 signaling and observed similar results (Figure 5D and 5E). Gab1 deficiency did not affect HGF receptor c-Met tyrosine phosphorylation (Supplemental Figure XIX). More-
are essential components in angiogenic processes in vivo. Taken in vitro studies showed that Gab1 is required for HGF-induced angiogenesis. In agreement with our in vivo results, our in vitro studies have shown that Gab1-ecKO mice underwent severe toe necrosis and had much lower capillary density in the ischemic limb than the nonischemic control, indicating that Gab1-ecKO mice not only have impaired angiogenesis but also show potential regression of preexisting vasculature. Therefore, we reasoned that a Gab1-mediated cell survival mechanism may maintain preexisting vasculature and regulate angiogenesis under ischemic conditions. Indeed, our TUNEL and vWF double staining studies demonstrate that there is significant EC death in the ischemic hindlimb of Gab1-ecKO mice. In particular, we detected vascular EC death in the ischemic hindlimb of Gab1-ecKO mice, starting as early as 2 days and persisting at 7 days after femoral artery resection. To our knowledge, this is the first evidence showing the potential role of EC survival in hindlimb ischemia-initiated angiogenesis and the role of Gab1 in EC survival in vivo. Consistent with these observations, we found that ECs isolated from Gab1-ecKO mice are more sensitive to starvation-induced cell death than those isolated from WT mice. The levels of cleaved caspase 3, a critical executor of apoptosis, were enhanced in ECs isolated from Gab1-ecKO mice under starvation conditions, further supporting the concept that Gab1 acts as a survival factor against EC death. Collectively, our results indicate that Gab1-mediated EC survival is involved in ischemia-initiated angiogenesis.

Akt and ERK1/2 activation have been shown to regulate cell migration and survival signaling pathways. Several studies have suggested that Akt and ERK1/2 pathways are involved in HGF-mediated angiogenesis in vitro. Gab1, an adaptor protein, plays an important role in mediating growth factor–induced activation of Akt and ERK1/2 through recruiting PI3K and SHP2 in a tyrosine phosphorylation–dependent manner. However, the role of Gab1 in HGF-mediated signaling in ECs remains unclear. We first found that HGF strongly stimulated Gab1 tyrosine phosphorylation in ECs. Using Gab1-deficient ECs isolated from Gab1-ecKO mice and Gab1 siRNA knocked-down HUVECs, as well as Gab1 phosphotyrosine mutants, we further found that Gab1-mediated HGF-induced Akt and ERK1/2 activation in ECs. The data provide signal mechanisms by which Gab1 mediates growth factor–induced angiogenesis.

Many growth factors are implicated in the processes of angiogenesis in vivo. Our results, showing that VEGF and HGF are induced in response to ischemia, suggest that both VEGF and HGF are involved in ischemia-induced angiogenesis. In vitro studies have shown that Gab1 is tyrosine phosphorylated and mediates angiogenesis in vitro in response to VEGF. Consistent with this concept, we found that VEGF-induced angiogenesis was affected in Gab1-ecKO mice. However, we observed much stronger Gab1 tyrosine phosphorylation in ECs in response to HGF than in response to VEGF. We further found that HGF-
Figure 4. Gab1 deficiency in ECs impaired microvessel sprouting, tube formation, and migration. A and B, Microvessel sprouting in aortic ring assay. Representative micrographs and statistical results of sprouting microvessels from aortic ring grown in the presence and absence of 100 ng/mL HGF after 7 days are shown. *\(P<0.05\) vs WT + HGF, \(n=4\). C and D, Tube formation. Gab1-knockout (Gab1-KO) ECs showed impaired HGF (10 ng/mL)–induced capillary-like tube formation in the Matrigel analyzed by in vitro tube formation assay. *\(P<0.05\) vs WT + HGF, \(n=4\). E, EC migration measured by the Boyden chamber method with Gab1-KO ECs and WT ECs. Bar graph represents averaged data, expressed as migrated cell number counted per 10 fields (magnification, ×200). *\(P<0.05\) vs WT + HGF, \(n=4\). F to J, EC migration measured by wound healing assay with Gab1-KO ECs and WT ECs (F and G) or Gab1-KO ECs infected with adenoviral (Ad) LacZ or Gab1 (H and J). Representative images and percentage increase in migrated cell number over that in unstimulated cells (control) are shown. *\(P<0.05\) vs WT + HGF, \(n=4\); #\(P<0.05\) vs LacZ + HGF, \(n=4\).
induced angiogenesis in vitro and in vivo was severely impaired in Gab1-ecKO mice. Our results suggest that Gab1 signaling is more important in HGF-mediated angiogenesis than in VEGF-mediated angiogenesis. It has been shown that enhanced secretion of cardiac HGF from myocardial infarct regions is associated with an attenuation of ventricular enlargement and an improvement in cardiac function.\(^3\)\(^7\) In fact, administration of recombinant HGF promotes angiogenesis in ischemic hindlimb and heart in various animal models.\(^6\),\(^3\)\(^8\)\(^3\)\(^9\) In this study, we uncovered an essential role for Gab1 in ischemia-, tumor-, and HGF-mediated angiogenesis. Our findings may have clinical implications: they suggest that blocking Gab1 function results in the inhibition of tumor angiogenesis and tumor growth and that enhancing Gab1 signaling could be a potential therapeutic strategy to improve ischemic diseases.

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**Figure 5. Gab1-mediated angiogenic and survival signaling in ECs.** A to C, Knockdown of Gab1 by siRNA in HUVECs inhibited HGF (10 ng/mL)-mediated time-dependent activation Akt and ERK1/2. *P*<0.05 vs control siRNA + HGF, n=3. D and E, Gab1-deficient ECs isolated from Gab1-ecKO mice had impaired Akt and ERK1/2 activation in response to HGF (10 ng/mL) for 10 minutes. *P*<0.05 vs WT+HGF, n=3. F to H, Overexpression of Gab1 mutants in HUVECs affected Akt and ERK1/2 activation in response to HGF (10 ng/mL) for 10 minutes. *P*<0.05 vs WT+HGF, n=3.
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Supplemental Materials and Methods

Genotyping and PCR primers
Gab1-ecKO mice were genotyped using the following primers:

**Gab1:**
5'-GGTGAATCGACGGGTGCTTGTGA-3'
5'-CAGATTGGCCTTTGAACTGGAAG-3'

**Cre:**
5'-GCCTGCATTACCGGTCGATGCAACGA-3'
5'-GTGGCAGATGCGCGCACTGATT-3'

VEGF and HGF were detected using the following primers:

**VEGFA:**
5'-GGAGAGCAGAAGTCCCATGA-3'
5'-ACACAGGACGGCTTGGAAGAT-3'

**HGF:**
5'-AACACACTGGCCTCTTCTATGGCT-3'
5'-TGCTCGACCTGCAATCCTGGATAA-3'

Visualization of retinal vessels
WT and Gab1-ecKO Mice were anesthetized with ketamine/xylazine (130/10 mg/kg in ~50ul), and perfused with saline containing 40 mg/mL FITC-labeled dextran (Sigma) through the left ventricle. Eyes were removed and fixed in 4% paraformaldehyde/PBS solution for 1 hr. Retinas were dissected and flat mounted, as previously described.1

Whole-mount visualization of hind-limb arteries by pigment particle perfusion
This method as described previously,2 allowed us to visualize the arterial domain in its entity. Briefly, mice perfusion-fixation procedure with 4% (wt/vol) paraformaldehyde through left ventricle was followed by perfusion with special gouache pigment-solution (Schmincke, cat. no. HKS 318) which does not enter the capillaries or veins. This leads to staining of all arteries and arterioles. A procedure of transparency induction (transparency solution is mixing equal volumes of benzyl benzoate, Sigma, cat. no. B-6630, and benzyl alcohol, Aldrich, cat. no. B1, 620-8) is carried out on whole muscles to increase the artery-to-tissue contrast. Then pictures were taken.

Hindlimb ischemia model
The hindlimb ischemia model was used as previously described.3 Briefly, 8-12-week-old male C57BL/6 background wt mice and gab1-ecKO mice were used for the experiments. Animals either underwent right femoral artery ligation or a sham operation. Mice were anesthetized with 1.5% Isoflurane with Vapor 19.1 Isoflurane Vaporizer (Draeger, France). A skin incision was performed on the medial aspect of the right thigh. After careful separation of the vein and nerve, the femoral artery was ligated and cut immediately distal to the inguinal ligament and proximal to the popliteal bifurcation site. No branches were ligated along the length of the excised segment. In the sham group, the femoral artery was dissected free but not ligated. After surgery, all animals were closely monitored and sacrificed at 2wks. The entire adductor muscle and
gastrocnemius muscle were used for histology staining. The study protocol was approved by University Committee on Animal Resource of University of Rochester.

**Laser Doppler perfusion imaging (LDPI)**
LDPI (Moor Instruments Ltd. Millwey Axminster, Devon, UK) was used to record perfusion of both right and left limbs at different time points as indicated. Excess hair was removed by depilatory cream from the limb before imaging. Mice were placed on a heating plate at 37°C to minimize temperature variation. Color-coded images were recorded.

**Capillary density analysis**
Capillary density was examined by counting the number of capillaries in light microscopic sections taken from the ischemic and non-ischemic limbs. The gastrocnemius from each animal was examined. Serial sections were cut at two different levels approximately 200 μm apart. On each series, CD31 staining was performed. Thirty fields of CD31 staining were counted from the two levels for each of 3 animals, for a total of 90 fields per time point. Capillaries were counted under a 40x objective to determine the capillary density (mean number of capillaries per fiber).

**TUNEL staining**
TUNEL staining was performed according to the manufacturer’s protocol of In Situ Cell Death Detection Kit from Roche (Cat. No. 11684817910).

**In vivo angiogenesis assay with Matrigel plug**
Mice were anaesthetized and injected subcutaneously into the flank with 10 mg/mL growth factor reduced Matrigel (BD Biosciences), mixed with HGF (100ng/mL) or VEGF (100ng/mL) and 15 units of heparin. After 6 days, the animals were euthanized and dissected to remove the Matrigel plugs. These implants were then processed and embedded with paraffin for future staining. For hemoglobin analysis, the Matrigel plug was harvested after 6 days and homogenized in 130 μl de-ionized water. After centrifugation, the supernatant was used in the Drabkin assay (Sigma-Aldrich) to measure hemoglobin concentration.

**Tumor angiogenesis**
Mice of 8-12 weeks were anesthetized with ketamine/zylazine(100/20 mg/kg in ~50ul). The fur in the flank region were removed using a fur trimmer (Wahl clipper corporation, Sterling) and the skin cleansed with 70% ethanol. Subcutaneous injection of 1 x 10⁶ mouse melanoma cells (B16F10) in 100ul serum-free cell culture medium was performed using a 1ml syringe fitted with a 25-gauge needle. Mice were sacrificed 10 days after the injection. Tumors were collected and processed for imaging, weight, and histology.

**Aortic ring assay for ex vivo angiogenesis**
Mouse aortic ring angiogenesis assays were performed as previously described. Briefly, thoracic aortas from WT and Gab1-ecKO mice were dissected and the periaortic fibroadipose tissue removed under a stereo dissection microscope using finetipped forceps and micro-dissection scissors. 1-mm long aortic rings were embedded in growth factor-reduced Matrigel supplemented with 20 U/mL heparin. The aortic rings were treated with or without 50 ng/mL HGF and incubated at 37°C, 5% CO₂ for 8 days for
optimal micro-vessel sprouting. Images were taken using an Olympus BX41 microscope (Olympus, Center Valley, PA).\(^6\)

**Isolation of Mouse Lung Endothelial Cells**

Four mice from each group were sacrificed by CO\(_2\) euthanasia. Lungs were cut out and minced. Lung pieces were transferred into 25mL of pre-warmed type I collagenase (2mg/mL) and incubated at 37°C with gentle agitation for 45 min. Suspension was triturated using a 30 cc syringe firmly attached to a cannula at least 12 times and pipetted through a 70µm disposable cell strainer (Falcon# 35 2350) into a 50mL conical tube. Cell suspension was centrifuged at 400g (1300rpm in GH 3.7 rotor) for 8 min at 4°C and resuspended in 2mL of cold DPBS with Ca/Mg+0.1BSA. The cell suspension was then incubated with 30ul PECAM-1 coated Dynabeads (Dynal Biotech cat. 110.07) on a rotor at room temperature for 15 min, washed till clear by adding 8 mL of medium, mixed and mounted in a magnetic separator and left for 1-2 min. Cells with beads were resuspended in 5mL growth medium and plated on gelatin or fibronectin coated 60mm dishes. When cells approached on confluence at 5-9 days after plating, a second sort of cells was performed. Cells were detached with trypsin/EDTA, and transferred to a 10mL conical tube and spun down at 400g for 4 min. The cell pellet was resuspended in 2mL of DPBS with Ca/Mg+0.1%BSA. 30ul PECAM-1 coated Dynabeads were added and incubated for 10 min at room temperature on rotator, followed by washing with magnetic separator. Dynabeads were then removed by trypsin digestion. Cells were resuspended in 5mL growth medium and plated in gelatin-coated 60mm dish.

**Cell culture and siRNA transfection**

HUVECs were isolated from fresh human umbilical veins and grown in Medium 200 with 5% FBS and LSGS (Invitrogen)\(^7\) and cultured in 60mm dishes. A mixture of Gab1 (AUACUUAGAUCUCGACUUAUU) or scramble siRNA at 100nM final concentration and Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) were spotted into each dish and incubated for 2 hours followed by full culture medium change.\(^8\) Forty-eight hours later, experiments were performed on siRNA transfected HUVECs.

**Wound closure cell migration**

Mouse lung endothelial cells isolated from WT and Gab1-ecKO mice were cultured on 6-well plates coated with gelatin and grown to confluence. Cells were treated with HGF (10ng/mL). Monolayers were then disrupted with a cell scrapper of ~1.2 mm and photographed at 0 h and 12 h after HGF addition with a light microscope (Olympus CK40) equipped with a digital camera (Olympus DP11).\(^6\)

**Capillary-like tube formation assay for in vitro angiogenesis**

For *in vitro* angiogenesis assay, mouse lung endothelial cells were plated on a thin layer of Matrigel (BD Biosciences) at 5×10\(^4\) cells/well of a 24-well plate in 1% FBS EC medium and incubated for 12 h at 37°C. The capillary-like tube structures were visualized by a light microscope (Olympus CK40) at different time points and imaged with digital camera (Olympus DP11).\(^6\)

**Adenovirus constructs and infection**

Adenovirus constructs encoding Gab1-WT, Gab1-ΔPI3K, and Gab1-ΔSHP2 were generated using ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer’s protocol. Adenovirus containing LacZ was used as a control. The infection of endothelial cells with recombinant adenovirus was performed as described previously.\(^6\)
**Western blot analysis**
Cells were harvested in lysis buffer and clarified by centrifugation. The protein concentrations in the lysates were determined using the Bradford method (BioRad, Hercules, California). Protein lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies. After incubating with fluorescence-conjugated secondary antibodies, immunoreactive proteins were visualized by Odyssey Infrared Imaging System (LI-COR Biotechnology, Nebraska). Densitometric analyses of immunoblots were performed with Odyssey software (LI-COR Biotechnology). Results were normalized by arbitrarily setting the densitometry of control sample to 1.0.

**Immuno-florescent staining and microscopy**
Cells were washed with PBS and fixed with 3.7% formaldehyde in PBS followed by washing with PBS three times at room temperature. Cells were then permeabilized with 0.5% Triton-X100 containing PBS for 10 min followed by three times washing with PBS and 10% normal goat serum of PBS as blocking buffer. Cells were incubated with appropriate primary antibodies. After three times washing with PBS, fluorescence-conjugated secondary antibodies were incubated and followed by three times washing with PBS. Images were captured by a fluorescence microscope (Olympus BX51, Olympus, Japan; Magnification: 60x; Numerical aperture: 0.90; Camera: RT Color, Model 2.2.1, Diagnostic Instruments Inc., Sterling Heights, MI; Software: SPOT Imaging software advanced, Diagnostic Instruments Inc., Sterling Heights, MI) or confocal microscope (Olympus; FLUOVIEW300).

**Cell starvation and MTT viability assay**
For starvation, cell culture media were changed to serum free media and incubated for 24 hours. Assessment of cell viability was performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Sigma cat. M5655). 0.5 mg/mL MTT was added to each well and cells were incubated for 4 h at 37°C. Cells were washed with PBS and lysed 30 min at room temperature with lysis buffer. Absorbance at 550 nm was photometrically measured.

**Data Analysis**
All data present the mean plus or minus standard error of the mean of at least three separate experiments. Differences between groups were tested for statistical significance using Student $t$ test or analysis of variance. Statistical significance was set at $P$ less than 0.05.
References


Supplemental Table I

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<th>Genotype</th>
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Supplemental Table I. Analysis of Progeny from male Tie2-Cre;Gab1^{floxed}/+ mice and female Gab1^{floxed}/floxed intercross.
Supplemental Figure I. Genotypes of Gab1-ecKO mice. Endothelium-specific Gab1 knockout mice (Gab1-ecKO, Tie2-Cre; Gab1\textsuperscript{flox/flox}) were generated by cross-breeding male Tie2-Cre; Gab1\textsuperscript{flox/+} mice with female Gab1\textsuperscript{flox/flox} mice. Representative image shows the results of genotypes by genomic DNA PCR method.
Supplemental Figure II. Morphology of lung endothelial cells isolated from Gab1-ecKO mice. Endothelial cells were isolated from pooled lungs of mice with Dynabeads conjugated with sheep-anti-mouse PECAM1 antibody. After two rounds sorting, the images of endothelial cell morphology were captured by a phase-contrast light microscopy. There were no obvious morphological difference between endothelial cells isolated from WT and Gab1-ecKO mice.
Supplemental Figure III. Positive PECAM1 and VE-Cadherin staining in lung endothelial cells isolated from Gab1-ecKO mice. The purity of mouse lung endothelial cells from isolated from WT and Gab1-ecKO mice was evaluated by endothelial marker PECAM1 staining (red) and VE-Cadherin (green) using anti-PECAM1 and anti-VE-Cadherin antibodies following nuclear DAPI counter staining (blue).
Supplemental Figure IV. Gab1 deficiency in lung endothelial cells isolated from Gab1-ecKO mice. Western blots analysis with Gab1, Gab2, and Gab3 antibodies showed that the level of Gab1, but not Gab2 and Gab3, expression in endothelial cells isolated from Gab1-ecKO mice was significant decreased compared to that isolated from WT mice. House keeping gene GAPDH serves as an internal control.
Supplemental Figure V. Endothelial specific Gab1 deficiency in gastrocnemius muscle tissues. Representative Gab1 and vWF double staining from sections of the gastrocnemius muscles in WT and Gab1-ecKO mice. Green: Gab1; Red: vWF positive. Note: Gab1 expression is more abundant in ECs than in muscle fibers.
Supplemental Figure VI. Hindlimb vasculature in WT and Gab1-ecKO mice.
Supplemental Figure VII

Supplemental Figure VII. Retina vascular network in WT and Gab1-ecKO mice.
Supplemental Figure VIII. Gab1-ecKO mice have enhanced vascular EC death in ischemic limb muscle. Representative TUNEL, smooth muscle alpha-actin, and vWF triple staining for endothelial cell death from sections of the gastrocnemius muscles at 2 days after femoral resection in WT and Gab1-ecKO mice. Green: TUNEL positive; Blue: smooth muscle alpha-actin; Red: vWF positive.
Supplemental Figure IX. VEGF and HGF expression in gastrocnemius muscle after hindlimb ischemia in WT and Gab1-ecKO mice. Total RNA of gastrocnemius muscles were extrated before or 1 day after femoral artery resection in WT and Gab1-ecKO mice. VEGF and HGF expression were detected by RT-PCR. * P < 0.05 vs WT 1 day, n=4.
Supplemental Figure X. HGF time-dependently stimulated Gab1 tyrosine phosphorylation in endothelial cells. HUVECs were exposed to HGF (10ng/mL) at indicated times. The cell lysates were subjected to immunoprecipitation with Gab1 antibody (Millipore) followed by Western blots with anti-phosphotyrosine-specific antibody 4G10 (Santa Cruz). The blots were then reprobed with Gab1 antibody for normalization. Representative blots and quantitative data are shown. * P < 0.05 vs control (0 min), n=3.
Supplemental Figure XI. Gab1 deficiency leads to impaired VEGF-mediated endothelial cell survival. VEGF-mediated mouse lung ECs isolated from WT and Gab1-ecKO mice survival under starvation conditions were analyzed by MTT cell viability assay. *P < 0.05 vs WT + starvation; **P < 0.05 vs WT + starvation + VEGF (10 ng/mL), n=4.
Supplemental Figure XII. Gab1 deficient ECs have increased cleaved caspase 3 expression under starvation conditions for 24 hours. *P < 0.05 vs WT + starvation, n=3.
Supplemental Figure XIII. Impaired microvessel sprouting in Matrigel plug assay in Gab1-ecKO mice. Representative micrographs of vessels with CD31 staining in the Matrigel in the presence or absence of 100 ng/mL VEGF after 7 days. Magnification, ×200. *P<0.05 vs WT, n=5.
Supplemental Figure XIV. Exogenous tumors derived from Gab1-ecKO mice are reduced in mass and volume. B16F10 melanoma cells were injected subcutaneously into the flanks of WT or Gab1-ecKO mice. Twelve days after injection, the tumors were excised from each mouse. A, Tumor gross appearance. Representative examples are shown. B, Tumor weight. C, Tumor volume. *P < 0.05 vs WT, n=6.
Supplemental Figure XV. Exogenous tumors derived from Gab1-ecKO mice are reduced vessel density. A-C, Tumor vessel density. Tumor paraffin-embedded sections were stained with CD31 antibody (arrow). Vessel number was determined by the number of continuous CD31-positive structures per field. Vascular density of tumors was quantified with the percentage of each field occupied by a CD31-positive signal.
Supplemental Figure XVI. Impaired migration of Gab1 deficient ECs. EC migration measured by the Boyden chamber method with WT and Gab1-KO ECs. Bar graph represents averaged data, expressed as fold increase of migrated cell. *P < 0.05 vs WT + VEGF, n=4.
Supplemental Figure XVII. Gab1 deficiency does not affect HGF- and VEGF-induced receptor tyrosine phosphorylation. ECs from WT and Gab1-ecKO mice were treated with HGF (10ng/mL) or VEGF (10ng/mL) for 10 min. c-Met and VEGFR2 tyrosine phosphorylation were detected using pY1234/Y1235-c-Met and pY1175-VEGFR2 antibodies (Cell Signaling), n=3.