Docking Protein Gab1 Is an Essential Component of Postnatal Angiogenesis After Ischemia via HGF/c-Met Signaling

Wataru Shioyama, Yoshikazu Nakaoka, Kaori Higuchi, Takashi Minami, Yoshiaki Taniyama, Keigo Nishida, Hiroysau Kidoya, Takashi Sonobe, Hisamichi Naito, Yoh Arita, Takahiro Hashimoto, Tadashi Kuroda, Yasushi Fujo, Mikiyasu Shirai, Nobuyuki Takakura, Ryuichi Morishita, Keiko Yamauchi-Takahira, Tatsuhiko Kodama, Toshio Hirano, Naoki Mochizuki, Issei Komuro

Rationale: Grb2-associated binder (Gab) docking proteins, consisting of Gab1, Gab2, and Gab3, have crucial roles in amplification and integration of signal transduction evoked by growth factors such as extracellular signal-regulated kinase (ERK)1/2 and AKT, and for HGF-induced stabilization of ECs via ERK5. In contrast, Gab1-p85 complex regulated activation of AKT and contributed partially to migration of ECs after HGF stimulation. Microarray analysis demonstrated that HGF upregulated angiogenesis-related genes such as KLF2 (Krüppel-like factor 2) and Egr1 (early growth response 1) via Gab1-SHP2 complex in human ECs. In Gab1ECKO mice, gene transfer of vascular endothelial growth factor, but not HGF, improved blood flow recovery and ameliorated limb necrosis after HLI.

Objective: To elucidate the role of Gab proteins in postnatal angiogenesis.

Methods and Results: Endothelium-specific Gab1 knockout (Gab1ECKO) mice were viable and showed no obvious defects in vascular development. Therefore, we analyzed a hindlimb ischemia (HLI) model of control, Gab1ECKO, or conventional Gab2 knockout (Gab2KO) mice. Intriguingly, impaired blood flow recovery and necrosis in the operated limb was observed in all of Gab1ECKO, but not in control or Gab2KO mice. Among several proangiogenic growth factors, hepatocyte growth factor (HGF) induced the most prominent tyrosine phosphorylation of Gab1 and subsequent complex formation of Gab1 with SHP2 (Src homology-2–containing protein tyrosine phosphatase 2) and phosphatidylinositol 3-kinase subunit p85 in human endothelial cells (ECs). Gab1-SHP2 complex was required for HGF-induced migration and proliferation of ECs via extracellular signal-regulated kinase (ERK)1/2 pathway and for HGF-induced stabilization of ECs via ERK5. In contrast, Gab1-p85 complex regulated activation of AKT and contributed partially to migration of ECs after HGF stimulation. Microarray analysis demonstrated that HGF upregulated angiogenesis-related genes such as KLF2 and Egr1 via Gab1-SHP2 complex in human ECs. In Gab1ECKO mice, gene transfer of vascular endothelial growth factor, but not HGF, improved blood flow recovery and ameliorated limb necrosis after HLI.

Conclusion: Gab1 is essential for postnatal angiogenesis after ischemia via HGF/c-Met signaling. (Circ Res. 2011; 108:00-00.)

Key Words: angiogenesis | Gab1 | growth factors | endothelial cells | signal transduction

The Grb2-associated binder (Gab) family docking proteins, consisting of Gab1, Gab2, and Gab3, are involved in amplification and integration of signal transduction evoked by growth factors, cytokines, antigens, and numerous other molecules.1,2 Gab proteins lack enzymatic activity but become phosphorylated on tyrosine residues, providing binding sites for multiple SH2 (Src homology-2) domain-containing proteins such as SHP2 (SH2-containing protein tyrosine phosphatase 2), phosphatidylinositol 3-kinase regulatory subunit p85, phospholipase Cγ, Crk, and GC-GAP. Docking of Gab proteins to SHP2 and p85 is considered to be essential for activation of mitogen activated protein kinase (MAPK), such as extracellular signal-regulated kinase (ERK)1/2 and AKT, respectively.1,2 Conventional Gab1 knockout (Gab1KO)
mice display embryonic lethality with impaired development of heart, placenta, skin, and skeletal muscle. Conventional Gab2 knockout (Gab2KO) mice do not show any obvious developmental defects, but display impaired allergic responses, osteoclast defects, and abnormal hematopoiesis in adulthood. Gab3 knockout mice exhibit no obvious phenotype. Because Gab1KO mice are embryonic lethal, we and others created conditional knockout mice of Gab1 using the Tie2-Cre system. We created cardiomyocyte-specific Gab1/Gab2 double knockout mice and reported that Gab1 and Gab2 have the redundant roles for maintenance of cardiac function via neuregulin-1/ErbB signaling.

Angiogenesis, the process of new blood vessel formation, is involved in many physiological and pathological settings such as ischemia, atherosclerosis, diabetes, and cancer. During angiogenic growth, some endothelial cells (ECs) within capillary vessel wall are selected for "sprouting" and acquire invasive and motile behaviors. The tip cells, which lead the growing sprout, are guided by vascular endothelial growth factor (VEGF) gradients. The migration and proliferation of ECs behind the tip promote sprout extension. Fusion of ECs at the EC-EC interfaces establish a continuous endothelial cell syncytium. Extracellular signal-regulated kinase (ERK)1/2, ERK5, and AKT after stimulation with HGF in ECs. Gab1, but not Gab2, is required for activation of ERK1/2, ERK5, and AKT after stimulation with HGF in ECs. Gab1-SHP2 complex positively regulates migration and proliferation of ECs via ERK1/2 and contributes to stabilization of ECs via ERK5 presumably in association with upregulation of KLF2.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Animals

Gab1flox mice were generated in 129/Sv-C57BL/6J mixed background as described previously. Tie2-Cre transgenic mice in CD-1 background were provided from Dr Thomas N. Sato. Gab1ECKO mice were generated by crossing Gab1flox/flox mice with Tie2-Cre transgenic mice. The creation of Gab2KO (Gab2flox/flox Gab2−/−) mice were also described previously. All the animals used for the experiments were 7- to 8-week-old male mice maintained on a 129/Sv-C57BL/6j-C57BL/6j mixed background. We housed all animals in a virus-free facility on a 12-hour light/12-hour dark cycle and fed them a standard mouse food. All experiments were carried out under the guidelines of Osaka University Committee for animal and DNA experiments and were approved by the Osaka University Institutional Review Board.

### Results

**Generation of Endothelium-Specific Gab1 Knockout Mice**

To elucidate the functional role of Gab1 in the endothelium, we first generated endothelium-specific Gab1 knockout mice (Gab1ECKO) using the Cre-loxP system. We created a Gab1flox allele by introducing 2 loxP sites into introns flanking exon 2 which encodes part of the pleckstrin homology domain as described previously. To cause recombination of the floxed allele exclusively in EC lineage, mice homozygous for the Gab1flox/flox-targeted allele (Gab1flox/flox) were crossed with transgenic mice expressing Tie2 promoter-driven Cre recombinase (Tie2-Cre mice). We created Gab1ECKO (Gab1flox/flox Tie2-Cre(+)) mice by crossing Gab1flox/flox Tie2-Cre(+) mice
with Gab1^{flox/flox} mice. The offspring of these crossings were obtained at expected Mendelian ratios as follows: Gab1^{flox/flox} Tie2-Cre(+) (n=23; 24.5%); Gab1^{flox/flox} (n=27; 28.7%); Gab1^{flox/flox} Tie2-Cre(+) (n=24; 25.5%); Gab1^{flox/flox} (n=20; 21.3%).

To confirm the knockout of Gab1 protein in the vascular endothelium, the CD31-positive ECs were purified from the limb muscles of control (Gab1^{flox/flox}) and Gab1ECKO mice using the Magnetic Cell Sorting (MACS) system (Miltenyi Biotec Inc). The lysates of either purified CD31-positive ECs or CD31-negative cells were subjected to immunoblotting analyses. We confirmed successful depletion of Gab1 protein in CD31-positive ECs derived from Gab1ECKO mice, but not from control mice (Figure 1A). We also confirmed that

Figure 1. Impaired blood flow recovery and angiogenesis in Gab1ECKO mice. A, Gab1 was successfully ablated in the ECs in Gab1ECKO mice. The CD31-positive ECs were purified from the limb muscles using the MACS system. Whereas the expression of both Gab1 and Gab2 in the CD31-negative cells was almost comparable between the 2 groups, the expression of Gab1 was exclusively depleted in the CD31-positive cells in Gab1ECKO, but not in control mice. The expression levels of both Gab2 and β-tubulin were comparable between 2 groups. B, HLI was induced and blood flow of ischemic (right) and nonischemic (left) limb were measured on gastrocnemius muscle before and on the indicated days after surgery using LDBF analyzer. Tissues were harvested on day 21. C, All of Gab1ECKO mice showed limb necrosis after HLI, whereas control mice displayed no necrosis. D, Gross morphology of the ischemic limb was assessed on day 21 after surgery. E, Representative LDBF images of a mouse HLI on day 1, 3, 7, 14, and 21 after surgery. Red represents greater flow; blue, less flow. F, Quantitative analysis of blood flow recovery after HLI expressed as ischemic (right) to nonischemic (left) LDBF ratio in control (n=9) and Gab1ECKO mice (n=9). Values are shown as means±SEM. **P<0.01 vs control. G, Representative CD31 staining of capillaries from sections of nonischemic and ischemic adductor muscles. Scale bar, 100 μm. H, Quantitative analysis of capillary density in control and Gab1ECKO mice (number per high-power field; ×400 magnification). Values are shown as means±SEM. *P<0.05, **P<0.01 for the indicated groups. I, Arteriogenesis was determined by barium sulfate casting followed by x-ray microangiography. Three weeks after femoral artery ligation, mice were anesthetized and subjected to barium sulfate perfusion. Collateral artery growth is significantly attenuated in Gab1ECKO mice compared with control mice as indicated by arrowheads.
Gab1 expression in CD31-negative cells was almost comparable between control and Gab1ECKO mice (Figure 1A). There was no significant difference in Gab2 expression between control and Gab1ECKO mice both in CD31-positive ECs and CD31-negative cells (Figure 1A).

Next, we examined whether Gab1ECKO mice show vascular developmental abnormalities by whole-mount immunohistochemical staining with anti-CD31 antibody. Gab1ECKO mice did not show any obvious vascular developmental defects both during embryogenesis and at 8 weeks of age compared with control mice (Online Figure I, A through H). In addition, we crossed control (Gab1<sup>flox/flox</sup>) mice with Gab2<sup>−/−</sup> mice to create Gab1<sup>flox/flox</sup> Gab2<sup>−/−</sup> mice, designated as Gab2KO mice. Gab2KO mice did not show any obvious vascular developmental defects at birth almost similarly as Gab1ECKO mice (data not shown).

**Gab1 in the Vascular Endothelium Is Essential for Postnatal Angiogenesis and Arteriogenesis After Ischemia**

To determine the role of Gab1 and Gab2 in postnatal angiogenesis, control, Gab1ECKO, and Gab2KO male mice were subjected to HLI that was created by unilateral femoral artery ligation and analysis at different time points as diagrammed in Figure 1B. From day 7 to 21 after surgery, all of Gab1ECKO mice showed various grades of limb necrosis, whereas no necrotic phenotypes were observed in control and Gab2KO mice (Figure 1C and 1D; Online Figure II, A and B). To precisely determine functional defects in Gab1ECKO mice, blood flow of ischemic and nonischemic limb perfusion were measured before and on 1, 3, 7, 14, and 21 days after surgery using laser Doppler blood flow (LDBF) analyzer. Blood flows on the basal condition and on day 1 after surgery were comparable among mice from each group. Compared with the nonischemic limb, blood flow recovery of the ischemic limb was also comparable between control and Gab2KO mice (Online Figure II, C and D). These findings indicate that Gab2 is not critically engaged in blood flow recovery after HLI. In clear contrast, blood flow recovery in Gab1ECKO mice was substantially impaired on 7, 14, and 21 days (Figure 1E and 1F). These results indicate that endothelial Gab1 has a crucial role for blood flow recovery in response to HLI.

The improvement in blood flow recovery mainly corresponds to increased tissue capillary densities on day 21 after HLI (Figure 1G and 1H). The capillary densities in the nonischemic adductor muscles were comparable between control and Gab1ECKO mice (Figure 1G and 1H). On the other hand, control mice showed increased capillary densities in the ischemic adductor muscles, whereas Gab1ECKO mice exhibited no significant increase in capillary densities (Figure 1G and 1H). These findings indicate that Gab1, but not Gab2, has an essential role for blood flow recovery via the angiogenic response to HLI.

We also examined ischemia-initiated arteriogenesis in control and Gab1ECKO mice by barium sulfate casting followed by x-ray angiographic analysis. Interestingly, Gab1ECKO mice showed a significantly attenuated collateral formation compared with control mice (Figure 1I). These data suggest that Gab1 might have a critical role not only in angiogenesis but also in arteriogenesis after HLI.

**HGF Induces the Strongest Tyrosine Phosphorylation of Gab1 and Gab2 in the ECs**

Several proangiogenic factors have been reported to regulate angiogenesis after ischemia. To elucidate how Gab1 is involved in the angiogenic response in the vascular endothelium, we performed in vitro experiments using human umbilical vein ECs (HUVECs). We first examined the expression of Gab family transcripts by RT-PCR and detected the mRNA of Gab1 and Gab2, but not that of Gab3 in HUVECs and human aortic ECs (Figure 2A). To examine which ligand induces tyrosine phosphorylation of Gab1 in HUVECs, cells were stimulated with proangiogenic factors such as HGF, VEGF, and fibroblast growth factor (FGF2). Among these, HGF induced the strongest tyrosine phosphorylation of Gab1 and the subsequent complex formation of Gab1 with SHP2 and p85 in HUVECs (Figure 2B). We confirmed this result using 2 antibodies recognizing Gab1 only if phosphorylated on Tyr-627 or Tyr-307. Figure 2D and 2E show that both residues are strongly phosphorylated in response to HGF stimulation of HUVECs. We also examined the tyrosine phosphorylation of Gab2, another Gab family protein expressed in HUVECs, after stimulation with HGF, VEGF, or FGF2. HGF induced strong tyrosine phosphorylation of Gab2 and the subsequent complex formation of Gab2 with SHP2 and p85 in HUVECs, almost similarly as that of Gab1 (Figure 2C). Thus, Gab1 and Gab2 undergo strong tyrosine phosphorylation on HGF stimulation, suggesting that Gab1 and Gab2 might have a role for HGF-dependent signaling in HUVECs.

We also examined activation of downstream signaling pathways of Gab proteins after stimulation with HGF, VEGF, or FGF2. Among these, HGF induced the strongest and the most sustained activation of ERK1/2 and AKT in HUVECs (Figure 2D, 2F, and 2G). We previously reported that Gab1 is critically involved in activation of ERK5 after stimulation with leukemia inhibitory factor in cardiomyocytes. Therefore, we performed ERK5 in vitro kinase assay using glutathione S-transferase (GST) fusion protein containing transactivating domain of myocyte enhancer factor 2 (MEF2C) (GST-MEF2C) as a substrate. HGF induced the strongest activation of ERK5 in HUVECs among these agonists (Figure 2H and 2I). Collectively, HGF induces the strongest activation of ERK1/2, AKT, and ERK5 in HUVECs, indicating that Gab family proteins might have an important role for full activation of these downstream pathways in HUVECs.

**Gab1, But Not Gab2, Is Required for Activation of ERK1/2, AKT, and ERK5 After Stimulation With HGF in HUVECs**

To examine the role of Gab1 and Gab2 in HGF-dependent signaling pathway, we performed small interfering (si)RNA-mediated knockdown of Gab1 and Gab2 in HUVECs. We observed successful depletion of Gab1 or Gab2 protein in HUVECs 48 hours after transfection with the Gab1- or Gab2-specific siRNA, respectively (Figure 3A). The specificity of this inhibition was demonstrated by the unaltered expression of ERK1/2 and AKT in each condition (Figure 3A). HGF-induced activation of ERK1/2, AKT, and ERK5 were significantly attenuated in HUVECs transfected with Gab1-specific siRNA compared with those transfected with.
control siRNA (Figure 3A through 3E). Conversely, HGF-induced activation of ERK1/2, AKT, and ERK5 were significantly enhanced in HUVECs transfected with Gab2-specific siRNA compared with those transfected with control siRNA (Figure 3A through 3E), suggesting that Gab2 might exert an inhibitory role for HGF/c-Met/Gab1-dependent signaling. These data indicate that Gab1 and Gab2 might have an opposite role for activation of ERK1/2, AKT, and ERK5 after HGF stimulation in HUVECs.

**Gab1 Has an Essential Role for HGF-Dependent Signaling Through Association With SHP2 and p85 in HUVECs**

To delineate the role of Gab1 in HGF-dependent signaling, we used adenovirus vectors expressing β-galactosidase (β-gal) (control), wild-type Gab1 (Gab1WT), mutated Gab1 that is unable to bind SHP2 (Gab1ΔSHP2), or mutated Gab1 that is unable to bind p85 (Gab1Δp85), as described previously.18,24 We found that Gab1 indeed associated with c-Met after stimulation with HGF in HUVECs overexpressing Gab1WT (Online Figure III). Next, we examined the effect of adenovirus-mediated forced expression of Gab1WT, Gab1ΔSHP2, or Gab1Δp85 on the HGF-dependent downstream signaling pathways. HGF induced activation of ERK1/2, AKT, and ERK5 in the control HUVECs expressing β-gal (Figure 4A and 4D). Whereas HGF-induced activation of ERK1/2 was augmented in HUVECs expressing Gab1WT or Gab1Δp85 compared with control cells expressing β-gal, activation of ERK1/2 was significantly attenuated in HUVECs expressing Gab1ΔSHP2 (Figure 4A and 4B). Furthermore, HGF-induced activation of ERK5 was enhanced in HUVECs expressing Gab1WT compared with control cells expressing β-gal. In addition, enhanced activation of ERK5 activity was quantified by scanning densitometry and was expressed relative to input ERK5 (total cell lysate). The results were expressed as relative intensity over cells treated with vehicle. *P<0.05, **P<0.01 for the indicated groups; #P<0.01, §P<0.05 vs HGF-treated cells at the same time after stimulation. Values are shown as means±SEM for 3 separate experiments.

**Figure 2. Gab1 and its downstream signaling pathways are strongly activated after stimulation with HGF in HUVECs.** A, RT-PCR shows the expression of Gab1 and Gab2 mRNAs, but not Gab3 mRNA, in both HUVECs and human aortic ECs (HAECs). B and C, Tyrosine phosphorylation of Gab1 (B) and Gab2 (C) and their association with SHP2 and p85 were analyzed by immunoprecipitation of the HUVECs lysates. HUVECs were stimulated with HGF, VEGF, or FGF2 and cell lysates were subjected to immunoprecipitation with anti-Gab1 (B) or anti-Gab2 (C) serum, followed by immunoblotting analysis using the antibodies indicated at the left. D, Phosphorylation of Gab1 on Tyr-627 or Tyr-307, ERK1/2, and AKT were assessed by phosphor-specific antibodies. E, Phosphorylation of Gab1 on Tyr-627 was quantified against total Gab1 (n=3). F, Phosphorylation of ERK1/2 was quantified against total ERK1/2 (n=3). G, Phosphorylation of AKT (Ser473) was quantified against total AKT (n=3). H, ERK5 activity was measured by in vitro kinase assay using anti-ERK5 immunoprecipitates from the corresponding cell lysates as described in Methods (n=3). 32P-labeled substrates are shown at the top (GST-MEF2C). In parallel, cell lysates were subjected to immunoblotting with anti-ERK5 antibody (bottom) to confirm the equal amount loading. I, ERK5 activity was quantified against total ERK5 (total cell lysate) by scanning densitometry and was expressed relative to input ERK5 (total cell lysate). The results were expressed as relative intensity over cells treated with vehicle. *P<0.05, **P<0.01 for the indicated groups; #P<0.01, §P<0.05 vs HGF-treated cells at the same time after stimulation. Values are shown as means±SEM for 3 separate experiments.
against total AKT (n = 3). D, ERK5 activity was measured by in vitro kinase assay using anti-ERK5 immunoprecipitates from the corresponding cell lysates almost similarly described in Figure 2H (n = 3). 32P-labeled substrates are shown at the top (GST-MEF2C). In parallel, cell lysates were subjected to immunoblotting with anti-ERK5 antibody (bottom) to confirm the equal amount loading. E, ERK5 activity was quantified by scanning densitometry and was expressed relative to input ERK5 (total cell lysate). The results were expressed as relative intensity over cells treated with vehicle. **P < 0.01 for the indicated groups. Values are shown as means ± SEM for 3 separate experiments.

HGF Induces EC Migration Via Complex Formation of Gab1 With SHP2 and With p85

Next, we examined HGF-dependent EC migration as an in vitro model for the angiogenic response. HUVECs were infected with adenovirus vectors expressing β-gal, Gab1WT, Gab1ΔSHP2, or Gab1Δp85, and the effect of forced expression of various Gab1 proteins was examined in a monolayer “wound injury” assay. HGF-induced migration was significantly enhanced by overexpression of Gab1WT, but significantly repressed by that of Gab1ΔSHP2, compared with control cells expressing β-gal (Figure 4F and 4G). In addition, overexpression of Gab1Δp85 slightly reduced HGF-induced EC migration, compared with control cells (Figure 4F and 4G). These findings indicate that Gab1 regulates HGF-induced EC migration predominantly via complex formation with SHP2 and partly via that with p85.

To further delineate the downstream signaling pathways of Gab1-SHP2 complex responsible for HGF-induced EC migration, HUVECs were infected with adenovirus vectors expressing dominant-negative MAPK/ERK5 (MEK5DN), dominant-negative ERK5 (ERK5DN), or dominant-negative MAPK/ERK1 (MEK1DN). HGF-induced endothelial migration was almost abrogated by overexpression of MEK1DN, but not by that of MEK5DN or ERK5DN (Online Figure IV, A and B). In addition, we examined the effect of overexpression of constitutive-active MEK5 (MEK5CA) or constitutive-active MEK1 (MEK1CA) on the cell migration of HUVECs overexpressing Gab1ΔSHP2. Overexpression of MEK1CA, but not MEK5CA, restored cell migration of the HUVECs overexpressing of Gab1ΔSHP2 (Online Figure IV, C and D). Taken together, these findings indicate that MEK1/2-ERK1/2, but not MEK5-ERK5, is responsible for HGF-induced EC migration via Gab1-SHP2 complex.

HGF Stimulation Leads to a Distinct Pattern of Gene Expression via Gab1 in HUVECs

To explore the potential downstream target genes of HGF/c-Met/Gab1 signaling in the vascular endothelium, we used DNA microarrays to carry out a global survey of mRNA in HUVECs overexpressing various Gab1 proteins treated with or without HGF for 1 hour. Several transcripts were upregulated in response to HGF stimulation in the cells overexpressing Gab1WT, but not in those overexpressing either Gab1ΔSHP2 or Gab1Δp85 (Figure 5A). Because both Gab1-SHP2 and Gab1-p85 complex formation are prerequisite for HGF-induced EC migration as demonstrated in Figure 4F and 4G, we focused on these genes, which were upregulated by overexpression of Gab1WT, but not that of Gab1ΔSHP2 or Gab1Δp85, as presented in the cluster diagram (Figure 5A). By quantitative real-time RT-PCR, we confirmed that KLF2, Egr1 (early growth response 1), Egr3, and COX2 (cyclooxygenase-2) were indeed upregulated in HUVECs overexpressing Gab1WT, but not in those overexpressing Gab1ΔSHP2 (Figure 5B through 5E). Almost similar results were validated by immunoblotting analysis especially for the expression of KLF2 and Egr1 (Figure 5F).

KLF2 has important roles for vascular endothelial homeostasis downstream of several proangiogenic factors, laminar fluid shear stress, and statins. In addition, Egr1 has also been reported to be critical for ischemia-related gene regulation in the vascular endothelium. In addition, Egr1 has also been reported to be critical for ischemia-related gene regulation in the vascular endothelium. In addition, Egr1 has also been reported to be critical for ischemia-related gene regulation in the vascular endothelium.
antithrombotic function through KLF2/TM pathway down-stream of HGF/c-Met in the ECs.

To reveal the signaling pathways responsible for gene expression of KLF2 and Egr1, HUVECs were infected with adenovirus vectors expressing MEK5DN, ERK5DN, or MEK1DN. HGF-induced upregulation of KLF2 was almost abrogated by overexpression of either MEK5DN or ERK5DN, but not by that of MEK1DN, suggesting that HGF upregulates KLF2 gene via MEK5-ERK5 pathway (Online Figure V, B). Conversely, HGF-induced upregulation of Egr1 was suppressed by overexpression of MEK1DN, but not by overexpression of either MEK5DN or ERK5DN, suggesting that HGF induces upregulation of Egr1 through MEK1/2-ERK1/2 pathway (Online Figure V, C). These findings suggest that Gab1-SHP2 complex regulates HGF-induced upregulation of KLF2 and Egr1, via ERK5 and via ERK1/2, respectively.

**Gab1 Is Essential for HGF-Induced In Vivo Postnatal Angiogenesis**

We confirmed whether ischemia-induced angiogenesis was associated with a rise in HGF expression in the ischemic tissues. Ischemic tissues were harvested at the indicated time and subjected to ELISA. In control mice (Gab1flox/flox), a rise in HGF expression was observed in the ischemic tissues from 12 to 48 hours after HLI (Figure 6A). HGF expression levels in the ischemic limbs of control and Gab1ECKO mice were almost comparable at 24 hours after HLI by immunoblotting analysis (Online Figure VI, A and B). Almost similarly, VEGF expression levels in those of both control and Gab1ECKO mice were also almost similar at 24 hours after HLI (Online Figure VI, A and C).

We next evaluated the effect of HGF and VEGF gene transfer in HLI model in both control and Gab1ECKO mice. The vacant plasmid (pVAX1; control) and the expression plasmids of human HGF (pVAX1-HGF) and human VEGF165 (pVAX1-VEGF) were introduced after HLI as described in Methods. In control mice, injection of both pVAX1-HGF and pVAX1-VEGF plasmids into ischemic limbs significantly enhanced blood flow recovery on day 21 after HLI, compared with the pVAX1-injected group (Figure 6B and 6C). Intrigu-ingly, in Gab1ECKO mice, injection of pVAX1-VEGF into ischemic limbs significantly augmented blood flow recovery on day 14 and 21 after HLI, whereas injection of pVAX1-HGF did not increase blood flow recovery (Figure 6B and 6C). Consistent with these findings obtained from LDBF analysis, injection of pVAX1-VEGF rescued 60% of limb necrosis in Gab1ECKO mice, whereas injection of pVAX1-
HGF could only rescue 25% of limb necrosis in Gab1ECKO mice (Figure 6D). These data indicate that Gab1 is more strongly involved in HGF-dependent angiogenesis than in VEGF-dependent angiogenesis in vivo.

To validate the expression of downstream target genes of Gab1 in the endothelium, we purified CD31-positive ECs from both control and Gab1ECKO mice both at baseline and on day 1 after HLI. The expression of KLF2 and Egr1 in the vascular endothelium significantly decreased in Gab1ECKO mice compared with control mice, whereas the expression of CD31 and cyclophilin A was almost comparable between control and Gab1ECKO mice (Figure 6E through 6G). In addition, the expression of TM mRNA in the vascular endothelium significantly decreased in Gab1ECKO mice compared with control mice (Online Figure VII, A and B). Taken together, these findings suggest that HGF/c-Met/Gab1-dependent signaling was virtually attenuated both at baseline and after ischemia in the vascular endothelium of Gab1ECKO mice compared with control mice.
Discussion

The present study is the first to reveal that Gab1 in the endothelium is essential for in vivo angiogenesis after ischemia. Endothelium-specific deletion of Gab1 resulted in enhanced propensity of limb necrosis after HLI and impaired angiogenesis and arteriogenesis caused by the defect of HGF/c-Met signaling. Gab1 was engaged in activation of both ERK1/2 and ERK5 via association with SHP2 and in activation of AKT via association with p85 after stimulation with HGF in the ECs. Furthermore, we found that Gab1 regulates the expression of angiogenesis-related genes such as KLF2 and Egr1 in the vascular endothelium (Figure 7). Gab1, but not Gab2, was required for HGF-induced activation of ERK1/2, ERK5, and AKT in HUVECs, whereas both Gab1 and Gab2 underwent the most prominent tyrosine phosphorylation after stimulation with HGF among HGF, VEGF, and FGF2 (Figures 2 and 3). We found that siRNA-mediated knockdown of Gab2 in HUVECs leads to rather...
Figure 7. Schematic illustrations of the role of Gab1 in postnatal angiogenesis. HGF induces tyrosine phosphorylation of both Gab1 and Gab2 in the ECs. Both Gab1 and Gab2 associate with SHP2 and p85 on stimulation with HGF. Gab1 is required for activation of ERK1/2, ERK5, and AKT in response to HGF. Conversely, Gab2 has an opposite role as an endogenous inhibitor for activation of ERK1/2, ERK5, and AKT downstream of HGF/c-Met in the ECs. Gab2 might compete with Gab1 to become tyrosine-phosphorylated as a substrate for c-Met. After stimulation with HGF, Gab1-SHP2 complex positively regulates HGF-induced activation of both ERK1/2 and ERK5, leading to upregulation of Egr1 and KLF2, respectively. Gab1-SHP2 complex regulates EC migration via ERK1/2 pathway and EC stabilization via ERK5–KLF2 pathway after HGF stimulation. Gab1-p85 complex regulates HGF-induced activation of phosphatidylinositol 3-kinase/AKT pathway, which is partly responsible for EC migration. Furthermore, Gab1-p85 complex partially contributes to HGF-induced activation of ERK5 pathway. Collectively, Gab1 exerts an essential role for postnatal angiogenesis after ischemia via HGF/c-Met signaling.

Enhanced activation of ERK1/2, ERK5, and AKT in response to HGF (Figure 3). In EGF- or neuregulin-1–dependent signaling pathways, we and others previously reported that Gab2 can complement the loss of Gab1 for activation of ERK1/2 and AKT.11,28 In clear contrast, it has been reported that Gab1 is exclusively involved in HGF-dependent epithelial branching morphogenesis through activation of SHP2–ERK1/2 pathway in Madin–Darby canine kidney cells.29,30 Consistent with these findings, Gab1ECKO mice, but not Gab2KO mice, showed limb necrosis and impaired blood flow recovery after HLI, compared with control mice (Figure 1 and Online Figure II).

We demonstrated that HGF stimulation most strongly induced ERK5 activation among HGF, VEGF, and FGF2 in HUVECs (Figure 2). Gab1-SHP2 complex was required not only for ERK5 activation, but also for subsequent induction of KLF2 and TM after HGF stimulation in HUVECs (Figures 4 and 5; Online Figure V). Gab1-p85 complex was partly involved in both activation of ERK5 and subsequent induction of KLF2 and TM (Figures 4 and 5; Online Figure V). ERK5 has been reported to be indispensable for both embryonic vascular development and maintenance of vascular integrity in mature blood vessels.31–33 ERK5 regulates vascular integrity through flow-mediated transcriptional upregulation of KLF2 gene expression in the endothelium.19 KLF2 exerts various vasoprotective, antithrombotic, and anti-inflammatory actions through upregulation of TM and eNOS genes.16 Recently, KLF2 has been reported to have a crucial role for in vivo angiogenesis.34 We found that the expression levels of both KLF2 and TM were significantly downregulated in the ECs from Gab1ECKO mice compared with control both before and after ischemia (Figure 6 and Online Figure VII). Reduced expression levels of KLF2 and TM in the ECs of Gab1ECKO mice after ischemia might be partly attributed to the abnormal HGF/c-Met signaling in the endothelium. However, further investigation is needed to elucidate the causal relationship between the angiogenic defects of Gab1ECKO mice and the expression levels of KLF2 and TM.

The previous studies demonstrated that Gab1KO mice phenocopy HGF knockout (HGF-KO) or c-Met knockout (c-Met-KO) mice.35,36 During embryonic stage, all of Gab1KO, HGF-KO, and c-Met-KO mice share defective skeletal muscle formation attributable to the impaired migration of muscle progenitor cells from somites to limb bud and abnormal placental formation. On the other hand, these knockout mice do not show any obvious vascular developmental defects during embryogenesis. Gab1KO mice do not share the abnormalities in vascular development observed in both VEGF and VEGF receptor (VEGFR2; Flk1) knockout mice.37–40 In addition, we could not detect any obvious vascular developmental defects in Gab1ECKO mice both during embryogenesis and after birth (Online Figure I), indicating that Gab1 in the vascular endothelium is not involved in vasculogenesis. Gene transfer of VEGF, but not HGF, improved blood flow recovery and partially rescued the necrotic phenotypes of Gab1ECKO mice after HLI (Figure 6). These findings suggest that Gab1 is more strongly involved in HGF-dependent angiogenesis rather than in VEGF-dependent angiogenesis in the adulthood. Taken together, we conclude that Gab1 exerts an essential role in postnatal angiogenesis and arteriogenesis after ischemia via HGF/c-Met signaling.

Acknowledgments
We thank K. Yamamoto and N. Maruyama for technical assistance; M. Suto and M. Kato for secretarial assistance; A. Izumi for assistance with the microarray experiments; M. Shibuya (Tokyo Medical and Dental University) for providing human VEGF165 cDNA; T. Ueyama (Kyoto Prefectural University School of Medicine) for providing the adenovirus vectors expressing dominant-negative MEK1 and constitutive-active MEK1; E. Nishida (Kyoto University) for providing dominant-negative MEK5 cDNA; and M. Masuda (National Cerebral and Cardiovascular Center Research Institute) for providing anti-CD31 chicken antibody.
Sources of Funding
This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan (to I.K., T. Kuroda, and Y.N.); the Ministry of Health, Labour, and Welfare of Japan (to I.K.); Japan Heart Foundation Young Investigator’s Research Grant (to Y.N.); Suzuken Memorial Foundation (to Y.N.); Astellas Foundation for Research on Metabolic Disorders (to Y.N.); Senni Life Science Foundation (to Y.N.); Takeda Medical Research Foundation (to Y.N.); and Mochida Memorial Foundation for Medical and Pharmaceutical Research (to Y.N.).

Disclosures
None.

References

Novelty and Significance

What Is Known?
- Blood vessel formation or angiogenesis is a complex process that depends on the actions of various proangiogenic growth factors.
- Grb2-associated binder (Gab) family docking proteins, consisting of Gab1, Gab2, and Gab3, mediate signaling for a variety of growth factors and cytokines.
- Conventional Gab1 knockout mice display embryonic lethality and share the developmental defects in placenta and skeletal muscle with HGF and c-Met knockout mice.
- Hepatocyte growth factor (HGF) and its receptor c-Met have a crucial role for postnatal angiogenesis after ischemia.

What New Information Does This Article Contribute?
- Endothelium-specific Gab1 knockout (Gab1ECKO) mice show enhanced propensity to limb necrosis after hindlimb ischemia (HLI) caused by impaired angiogenesis.
- Gab1 is required for HGF/c-Met-dependent signaling and angiogenesis in the endothelial cells.
- Global deletion of Gab2, another Gab protein expressed in the vascular endothelium, does not lead to limb necrosis and impaired blood flow recovery after HLI compared with wild-type mice.
- Gab1 regulates the expression of angiogenesis-related genes such as Krüppel-like factor (KLF)2 and early growth response (Egr)1 downstream of HGF/c-Met signaling.

We hypothesized that the Gab family docking proteins in the endothelium have crucial roles in angiogenesis, because Gab proteins have been reported to amplify and integrate signal transduction of various growth factors and cytokines. We found that endothelium-specific deletion of Gab1, but not global deletion of Gab2, leads to impaired blood flow recovery and enhanced propensity to limb necrosis after HLI, suggesting that Gab1 is required for postnatal angiogenesis after ischemia. Among proangiogenic growth factors such as HGF, VEGF, and FGF2, HGF induced the strongest tyrosine phosphorylation of Gab1 in endothelial cells. Adenovirus-mediated overexpression and siRNA-mediated knockdown studies revealed that Gab1, but not Gab2, is required for activation of ERK1/2, ERK5, and AKT after stimulation with HGF in endothelial cells. We also found that Gab1 upregulates the angiogenesis-related genes such as KLF2 and Egr1 downstream of HGF/c-Met signaling. In vivo gene transfer of VEGF, but not HGF, significantly improved the blood flow recovery and partially rescued limb necrosis after HLI in Gab1ECKO mice, suggesting that Gab1 is more strongly involved in HGF-dependent angiogenesis rather than in VEGF-dependent angiogenesis. Taken together, these findings indicate that endothelial Gab1 is essential for postnatal angiogenesis after ischemia via HGF/c-Met signaling.
Docking Protein Gab1 Is an Essential Component of Postnatal Angiogenesis After Ischemia via HGF/c-Met Signaling

Wataru Shioyama, Yoshikazu Nakaoka, Kaori Higuchi, Takashi Minami, Yoshiaki Taniyama, Keigo Nishida, Hiroyasu Kidoya, Takashi Sonobe, Hisamichi Naito, Yoh Arita, Takahiro Hashimoto, Tadashi Kuroda, Yásushi Fujio, Mikiyasu Shirai, Nobuyuki Takakura, Ryuichi Morishita, Keiko Yamauchi-Takahara, Tatsuhiko Kodama, Toshio Hirano, Naoki Mochizuki and Issei Komuro

*Circ Res.* published online February 3, 2011;
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2011/02/03/CIRCRESAHA.110.232223

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/02/03/CIRCRESAHA.110.232223.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Online Supplement

Methods

Reagents, Antibodies, and Recombinant Protein

We generated anti-KLF2 monoclonal antibody against amino acids 2-34 of human KLF2 as described previously. Other antibodies were purchased as follows: anti-phospho-Gab1 (Tyr627), anti-phospho-Gab1 (Tyr307), anti-Gab1, anti-phospho-AKT (Ser473), anti-phospho-AKT (Thr308), anti-AKT, anti-phospho-p44/p42 (pERK1/2) (Thr202/Tyr204), anti-p44/p42 (ERK1/2), anti-Gab2, anti-c-Met antibodies for immunoblotting, and horseradish peroxidase-coupled sheep anti-mouse and anti-rabbit IgG from Cell Signaling Technology; antibodies recognizing phosphor-tyrosine (PY99), Egr1, VEGF, HGF, and SHP2 were from Santa Cruz Biotechnology Inc.; antibody against p85 was from Millipore; antibodies against β-tubulin (mouse monoclonal) and ERK5 (big mitogen activated protein kinase; BMK1) were from Sigma; rat monoclonal antibody against CD31 for immunohistochemical analysis was from BD Biosciences PharMingen; antibody against cyclophilin A antibody was from Enzo Life Sciences. The chicken antibody against CD31 for immunoblotting was kindly provided by Dr. M. Masuda (National Cerebral and Cardiovascular Center Research Institute). The use of anti-Gab1 and anti-Gab2 serum in immunoprecipitation (IP) was described previously. Medium M-199 and BSA (cell culture grade) were purchased from Invitrogen. Human recombinant HGF and VEGF were from Sigma. FGF2 (bFGF) was from Calbiochem. GST-MEF2C was prepared as described previously.
Construction of adenovirus vector and plasmids

The generation of adenovirus vectors expressing human Gab1\textsuperscript{WT}, Gab1\textsuperscript{ASHP2}, Gab1\textsuperscript{Δp85}, ERK5\textsuperscript{DN} were described previously \textsuperscript{3,4}. The adenovirus vectors expressing dominant-negative MEK1 (MEK1\textsuperscript{DN}) constitutive-active MEK1 (MEK1\textsuperscript{CA}) was kindly provided from Dr. T. Ueyama (Kyoto Prefectural University School of Medicine). In this study, we constructed the adenovirus vector expressing dominant-negative MEK5 (MEK5\textsuperscript{DN}). The plasmid vector containing MEK5\textsuperscript{DN} cDNA was provided by Dr. E. Nishida (Kyoto University). For adenovirus production, the cDNA sequence encoding MEK5\textsuperscript{DN} were subcloned into the shuttle plasmid pACCMVpLpA. Recombinant adenovirus was then obtained according to the homologous recombination system described elsewhere \textsuperscript{9}. The adenovirus vector expressing β-gal was used as a control. The human VEGF\textsubscript{165} cDNA was kindly provided from Dr. M. Shibuya (Tokyo Medical and Dental University) and was inserted into the multi-cloning site of pVAX1 plasmid. The expression plasmid (pVAX1-HGF) coding human HGF cDNA was described previously \textsuperscript{10}.

Isolation of mouse endothelial cells

Limb muscle tissues of 7- to 8-week-old mice were excised and subjected to purification of murine endothelial cells (ECs) using a magnetic cell sorting (MACS) system. Excised tissues were minced and digested with dispase II (Sankojunyaku). After centrifugation at 1,500 × g for 3 min at 15 °C, the
cell pellets were digested with collagenase type I (Wako). After centrifugation at 1,500 × g for 3 min at 15 °C, the cell pellets were suspended with PBS containing 4% fetal bovine serum. Cell suspensions were filtered. The cell debris on the filter was again digested with collagenase type II (Worthington) and filtered. The filtered cells were then incubated with rat anti-mouse CD31 antibody (BD Biosciences PharMingen), and murine ECs were isolated by MACS according to the manufacturer’s instructions, using goat anti-rat IgG microbeads (Miltenyi Biotec).

**HLI model and injection of plasmids**

Seven- to eight-week-old male mice were subjected to HLI induced by extensive excision of the unilateral femoral artery under anesthesia as described previously. Briefly, after anesthesia by pentobarbital 80 mg/kg, the right femoral artery was exposed under a stereomicroscope. The artery was then ligated both proximally and distally using 7-0 silk sutures, and the ligated vessels were resected between the ligatures without damaging the femoral nerve. Sham operations involved skin incision without femoral artery ligation. For gene transfer experiment, the mice subjected to HLI operation were injected with pVAX1, pVAX1-HGF, or pVAX1-VEGF plasmid using GenomeOne-Neo transfection reagent (Ishihara Sangyo) into the quadriceps muscle of the ischemic limb according to the manufacturer’s instructions. Under anesthesia by intraperitoneal injection of a mixture of ketamine 80 mg/kg and xylazine 8 mg/kg, blood flow recovery was measured using LDBF analyzer (Moor LD12-IR; Moor Instruments) before and on 1, 7, 14, and 21 days after surgery, in both the ischemic
and nonischemic hindlimbs of the same animal. The blood flow values were expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

**Whole-mount Immunohistochemistry**

The whole-mount immunohistochemistry of mice embryos using anti-CD31 monoclonal antibody was performed as described previously. In brief, embryos were enucleated and fixed in 4% paraformaldehyde for 4 h and incubated in methanol overnight at 4 °C. For staining, the rehydrated specimens were blocked PBSMT (2% skim milk and 0.1% TritonX-100 in PBS) containing 5% normal goat serum and 1% BSA for 2 h at room temperature, incubated with PBSMT containing anti-CD31 antibody overnight at 4 °C, then washed five times in PBSMT each for 1 h at 4 °C. Specimens were incubated with PBSMT containing Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen) overnight at 4 °C, then washed three times in PBSMT and twice in PBST each for 1 h at 4 °C. Embryos were flat-mounted and examined using a fluorescent microscope.

The whole-mount immunohistochemistry of retina and trachea from 8-week-old mice using anti-CD31 monoclonal antibody was performed as previously described. In brief, 8-week-old control and Gab1ECKO mice were humanely killed. Eyes and tracheas were enucleated, fixed in 4% paraformaldehyde for 2 h and incubated in methanol for 3 h at 4 °C, washed in PBS, blocked in PBSMT containing 5% normal goat serum and 1% BSA for 3 h, and stained with anti-CD31 antibody, followed by incubation with Alexa Fluor 488 secondary antibody. Retinas were isolated from the eyes.
Retinas and tracheas were flat-mounted, and examined using a fluorescence microscope.

**Immunohistochemical analyses using cryoprotected sections**

The control and ischemic thigh adductor skeletal muscles were collected and fixed with 4% paraformaldehyde on day 21 after surgery, cryoprotected with 30% sucrose, and frozen in OCT compound (Sakura). Cryosections (6 μm) were stained with rat monoclonal anti-CD31 antibody and horseradish peroxidase (HRP)-coupled secondary antibody. Fifteen random microscopic fields from 3 different sections per mouse were examined. Capillary density was expressed as the number of capillaries per high-power field (hpf) (magnification, ×400).

**Microangiography**

Mice were anesthetized with sodium pentobarbital (80 mg/kg) and heparinized. A catheter was inserted from the femoral artery into the abdominal aorta, and a solution consisting of 70% weight/volume barium sulfate suspended in 7% gelatin (weight volume solution in water warmed in a water bath to 60°C) was injected into the abdominal aorta. Mice were then immersed in ice to solidify the contrast agent. Microangiography was taken with an angiographic system (MFX-80HK, Hitex) consisting of an open type 1 μm microfocus X-ray source (L9191, Hamamatsu Photonics) and a 50/100 mm (2”/4”) dual mode X-ray image intensifier (E5877JCD1-2N, Toshiba) at 60 kV and 60 μA. Using this system, the sample could be observed at any desired geometrical magnifications.
Expression analysis of Gab proteins by RT-PCR in HUVECs and HAECs

Total RNAs were extracted from HUVECs and HAECs using TRIzol reagent (Invitrogen) and were subjected to semi-quantitative RT-PCR. The first strand cDNAs were PCR-amplified, using the following primer pairs: Gab1: 5’-GCATGGAAGAGGAGATGGTGTTGTGTGTTG-3’ and 5’-AGTAGCAGAGGATGATCTGCTGG-3’; Gab2: 5’-GAGAAGAAGTTGAGGCGCTA-3’ and 5’-TGGACCCACTTATTCCATGTC-3’; Gab3: 5’-GAGAGCCTCTCTTTACACG-3’ and 5’-GGCTGAAGCTGTGGGGTA-3’; GAPDH 5’-TGAAAGGTCGAGTAGGGATATTG-3’ and 5’-CATGTGGGCGCATGAGTCCACCAC-3’.

Cell culture, siRNA-mediated protein knockdown, and adenovirus infection

HUVECs and HAECs were purchased from Lonza and were grown in endothelial growth medium-2 (EGM-2) BulletKit (Lonza). HUVECs were used within the first 8 passages. HUVECs were placed on collagen-coated plates (IWAKI). Stealth siRNA targeting the gene indicated below were purchased from Invitrogen: human Gab1 (HSS-103901), human Gab2 (HSS114867). HUVECs were transfected with 50 nM siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. After incubation for 48 h, the cells were used for the experiments. HUVECs were infected with adenovirus vectors at multiplicity of infection of 20 for 12 h. Thirty-six h after infection, cells were used for experiments.
**Cell Lysis, Immunoprecipitation and immunoblotting**

After serum-starvation in medium 199 containing 1% BSA for 6 h, HUVECs were stimulated with 20 ng/mL HGF, 50 ng/mL VEGF, or 20 ng/mL FGF2 for the indicated periods. Cells were washed twice with ice-cold PBS and scraped off in lysis buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 3 mmol/L EDTA, 1% Nonidet P-40, 2 mmol/L sodium orthovanadate and protease inhibitor cocktail Complete (Roche Applied Science) as described previously. Cell lysates were collected from confluent 100-mm dishes and precleared by 15,000 \( \times \) g centrifugation for 15 min. For immunoprecipitation, the cleared lysates were rotationally incubated with 1 \( \mu \)L of either anti-Gab1 or anti-Gab2 serum and with 20 \( \mu \)L of protein A-sepharose (GE Healthcare) for 8 h at 4 °C. The antigen-antibody complexes were collected by centrifugation, washed three times with lysis buffer without protease inhibitor mixture, and boiled in standard electrophoresis sample buffer. All the proteins immunoprecipitated were then resolved by SDS-PAGE and subjected to immunoblotting using a standard procedure. Blots were developed using ECL system (GE Healthcare). For direct immunoblotting analyses, the crude cell lysates were collected from 35-mm dishes and subjected to 15,000 \( \times \) g centrifugation for 15 min. The precleared lysates were then resolved by SDS-PAGE and subjected to immunoblotting analyses.

**Measurement of ERK5 activity by in vitro kinase assay**
HUVECs cultured on 100-mm collagen-coated dishes (IWAKI) were stimulated with vehicle or the indicated growth factors for 15 or 60 min. Cells were then lysed at 4 °C in lysis buffer containing 25 mmol/L HEPES at pH 7.5, 0.3 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1% Triton X-100, and 1× protease inhibitor mixture. To measure the ERK5 activity, in vitro kinase assay was performed as described previously. Briefly, endogenous ERK5 was immunoprecipitated from aliquots of cell lysates with anti-ERK5 antibody at 4 °C for 3 h, and the immunocomplexes were recovered with protein A-Sepharose beads (GE Healthcare). Beads were washed three times with PBS containing 1% Nonidet P-40 and 2 mmol/L sodium vanadate, once with washing buffer containing 100 mmol/L Tris at pH 7.5 and 0.5 mol/L LiCl and once with kinase reaction buffer containing 12.5 mmol/L MOPS at pH 7.5, 12.5 mmol/L β-glycerophosphate, 7.5 mmol/L MgCl2, 0.5 mmol/L EGTA, 0.5 mmol/L sodium vanadate, and 0.5 mmol/L sodium fluoride. Samples were then resuspended in 20 μl of kinase reaction buffer containing 3 μg of GST-MEF2C, 1 μCi of [γ-32P]ATP, and 20 μmol/L cold ATP and incubated at 30 °C for 90 min. 32P-labeled substrates were separated by SDS-PAGE and detected by autoradiography.

Monolayer EC migration assay

HUVECs, cultured on 35-mm collagen-coated dishes (IWAKI), were infected with the indicated adenovirus vectors and cultured until they reached the monolayer confluent state. The cells were
serum-starved in medium 199 containing 1% BSA for more than 6 h and scratched by a regular 20 µL-pipette tip along the diameter of the dish as described previously 17. Immediately after scratching, the cells were treated with vehicle or HGF (50 ng/mL) for 24 h. The EC migration in culture was determined by measuring the cell counts in the indicated areas.

**Microarray analysis**

Gene expression in the HUVECs in response to HGF stimulation was analyzed by Affymetrix microarray hybridization. HUVECs were infected with adenovirus vectors expressing β-gal, Gab1WT, Gab1ASHP2, and Gab1A085. Cells were serum-starved in medium 199 containing 1% BSA for 8 h, and stimulated with 20 ng/mL HGF for 1 h. Total RNA was purified using TRizol reagent (Invitrogen) and reverse-transcribed to cDNAs. Biotin-labeled RNAs derived from cDNAs were fragmented according to the manufacturer’s instructions (Affymetrix). Preparation of cRNA and hybridization of probe on arrays were performed according to the manufacturer's instructions (Affymetrix). File maker software was used for analyzing genes that demonstrated identical patterns in two independent microarray experiments. Data were analyzed according to the minimum information about a microarray experiment (MIAME) rule. To identify the genes regulated by Gab1 in response to HGF, we picked up the genes which fulfill the two criteria: (1) the hybridization signal (average difference) after HGF stimulation was higher than 100 and (2) the induction was greater than 1.5-fold in the cells overexpressing Gab1WT upon HGF stimulation. The genes that conformed to the two criteria were
further clustered on the basis of similar regulation patterns using Gene cluster and Java Tree View software according to the manual on the default settings.

**Real-time Reverse Transcription-PCR**

HUVECs infected with the indicated adenovirus vectors were serum-starved in medium M199 containing 1% BSA (Invitrogen) for 8 h and stimulated with 20 ng/mL HGF for 1 h. After stimulation, total RNA was purified using TRIzol reagent (Invitrogen). Quantitative real-time reverse transcription (RT)-PCR was carried out using QuantiFast SYBRGreen RT-PCR kit (Qiagen) as described before 18. For each reaction, 80 ng of total RNA was transcribed for 10 min at 50 °C followed by a denaturing step at 95 °C for 5 min and 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Fluorescence data were collected and analyzed using ABI PRISM 7900HT. The primers used for amplification of total RNA from HUVECs were as follows:  

- **KLF2**  
  5’-CTCACCAAGAGTTCGCTG-3’, 5’-CCGTGTGCTTTCCGTAAGT7G-3’;  
- **Egr1**  
  5’-TGACCCTGAGTCTTTTCCTT-3’, 5’-TGGGGTTGGTCTGTGACTC-3’;  
- **GAPDH**  
  5’-ATGGGGGAAGGGTGAAGGTCG-3’, 5’-GGGGTCTGCACTGCT-3’;  
- **Egr3**  
  5’-AGGAAAACCTATTGGGGAAAGTG-3’, 5’-GGAGCAATGAAATGTGGGTG-3’;  
- **COX2**  
  5’-GAATCATTACCAGGCAAATTG-3’, 5’-TCTGACTGCGGGGGAACACAA-3’;  
- **TM**  
  5’-TGTGGAATTGGGAGCCTGAG-3’, 5’-TCTCAGACTGGATGGA-3’. The primers used for amplification of RNA from the purified murine ECs were as follows:  

- **KLF2**  
  5’-CTGATGACCTCGCAACAGA-3’,
Quantification of HGF and VEGF levels in murine limb tissues

For quantification of HGF in control (Gab1\textsuperscript{lox/lox}) mice after HLI, the ischemic limb muscles were excised from control mice at the indicated day after operation. Tissue sample were extracted using the extraction buffer for mouse/ rat HGF (Institute of Immunology, Tokyo, Japan). The samples were subjected to quantification using the mouse HGF EIA kit (Institute of Immunology, Tokyo, Japan) according to the manufacturer’s instructions. To determine the expression level of both HGF and VEGF in the ischemic tissues, the ischemic limb muscles from control and Gab1ECKO mice were homogenized in lysis buffer containing 50 mmol/L HEPES, 100 mmol/L sodium fluoride, 2 mmol/L sodium orthovanadate, 4 mmol/L EDTA, 1% Tween-20, 0.1% SDS, and a protease inhibitor cocktail Complete using a Polytron homogenizer as described previously\textsuperscript{5}. The lysates were cleared by centrifugation at 14,000 g for 30 min. Protein concentration was measured with BCA protein assay kit (Pierce). The cleared lysates were subjected to immunoblotting following standard procedures as described previously\textsuperscript{4}.  

5'-GTGTTGTCACACACGAAGC-3'; \hspace{1cm} Egr1: \hspace{1cm} 5'-AGGAAAACCTATGGGAATG-3',

5'-GGAGCAATGAAATGTGGGTG -3'; \hspace{1cm} GAPDH : \hspace{1cm} 5'- TCTCCACACCTATGGTGCAA-3',

5' -CAAGAAACAGGGGAGCTGAG-3'; \hspace{1cm} TM: \hspace{1cm} 5'- TCTCCACACCTATGGTGCAA-3',

5'-CAAGAAACAGGGGAGCTGAG-3'; \hspace{1cm} CypA: \hspace{1cm} 5'-TTGTGGAATTGGGAGCTTG-3',

5' -TCTCATGAACTGGATGGGTG-3'.

11
Statistics

All data were expressed as means ± SEM. Differences among multiple groups were compared by one-way ANOVA followed by a post hoc comparison tested with Scheffe’s method. Student’s $t$-test was used to analyze differences between two groups. A value of $P < 0.05$ was considered as statistically significant.
Reference List


Online Figure I: Gab1ECKO mice display no obvious vascular developmental defects. (A, B) Whole-mount immunohistochemical staining of control and Gab1ECKO mice embryos at E10.5 with anti-CD31 antibody. No obvious difference between control (A) and Gab1ECKO (B) mice in vascular development was observed. Scale bar indicates 300 μm. (C-H) Comparison of retinal and tracheal blood vessels in 8-week-old control and Gab1ECKO mice. Whole-mount retina vasculature was stained with anti-CD31 antibody in control (C) and Gab1ECKO (D) mice. (E) and (F) are higher magnifications of the areas indicated by box in (C) and (D), respectively. Scale bar indicates 300 μm. Whole-mount trachea vasculature was stained with anti-CD31 antibody in control (G) and Gab1ECKO (H) mice. Scale bar indicates 100 μm.
Online Figure II: Deletion of Gab2 does not lead to limb necrosis and impaired blood flow recovery after HLI. (A) Gab2KO mice displayed no limb necrosis after HLI. (B) Gross morphology of the ischemic limb was assessed on day 21 after surgery. (C) Representative LBDF images of a mouse HLI on the indicated days after surgery. Red represents greater flow; and blue, less flow. (D) Quantitative analysis of blood flow recovery after HLI expressed as ischemic (right) to nonischemic (left) LDBF ratio in control (n=9) and Gab2KO mice (n=8). No significant difference was observed between the two groups. Values are shown as means ± SEM.
Online Figure III: Gab1 is recruited to the c-Met receptor after HGF stimulation in HUVECs. HUVECs infected with adenovirus vector overexpressing Gab1WT were serum-starved and stimulated with or without HGF (20ng/mL) for 15 min. Cell lysates were subjected to immunoprecipitation with either anti-Gab1 or anti-c-Met antibody, followed by immunoblotting analysis using the antibodies indicated at the left. To confirm equal amount loading, total cell lysates were also subjected to immunoblotting analysis with anti-Gab1 antibody.
Online Figure IV: HGF induces endothelial cell migration predominantly via MEK1/2-ERK1/2 pathway. (A) HUVECs infected with the indicated adenovirus vectors were serum-starved and subjected to “wound injury” assay by scratching. Cells were treated with or without HGF (50ng/mL) for 24 h. HGF-induced EC migration was significantly repressed by overexpression of MEK1DN. (B) Quantification for EC migration in “wound injury” assay. **P<0.01 vs β-gal. Values are shown as means ± SEM for three separate experiments. (C) HUVECs were subjected to dual infection of adenovirus vectors expressing either β-gal, MEK5CA, or MEK1CA with Gab1ΔSHP2, respectively. HUVECs were serum-starved and subjected to “wound injury” assay by scratching. Cells were treated with or without HGF (50ng/mL) for 24 h. HGF-induced EC migration was significantly enhanced by co-overexpression of Gab1ΔSHP2 and MEK1CA. (D) Quantification for EC migration in “wound injury” assay. **P<0.01 vs β-gal. Values are shown as means ± SEM for three separate experiments.
Online Figure V: HGF stimulation leads to a distinct pattern of gene expression via Gab1 in HUVECs. 
(A) HUVECs infected with the indicated adenovirus vectors were serum-starved and treated with vehicle or HGF (20 ng/mL) for 1 and 4 h. Total RNA was purified from the HUVECs and the expression levels of TM was analyzed by real-time RT-PCR as described in Figure 5 B-E. Bar graphs show relative RNA levels of each gene normalized to GAPDH levels. RNA levels are expressed relative to that in cells expressing β-gal treated with vehicle. The expression of TM mRNA was enhanced at 4 h after stimulation with HGF in HUVECs overexpressing Gab1\textsuperscript{WT} or Gab1\textsuperscript{Δp85}, but inhibited in cells expressing Gab1\textsuperscript{ΔSHP2}, compared with control cells expressing β-gal. (B and C) Gab1 regulates HGF-dependent upregulation of KLF2 and Egr1 genes via MEK5-ERK5 pathway and MEK1/2-ERK1/2 pathway, respectively. HUVECs infected with adenovirus vectors expressing β-gal, MEK5\textsuperscript{DN}, ERK5\textsuperscript{DN}, and MEK1\textsuperscript{DN} were serum-starved and treated with vehicle (-) or 20 ng/mL HGF (+) for 1 h. The expression levels of KLF2 (B) and Egr1 (C) mRNA was analyzed as described in Figure 5 B-E. HGF-induced upregulation of KLF2 mRNA was abrogated in HUVECs overexpressing MEK5\textsuperscript{DN} or ERK5\textsuperscript{DN}, but not in those expressing MEK1\textsuperscript{DN}. On the contrary, HGF-induced upregulation of Egr1 mRNA was abrogated in HUVECs overexpressing MEK1\textsuperscript{DN}, but not in those expressing MEK5\textsuperscript{DN} or ERK5\textsuperscript{DN}. **P<0.01, *P<0.05 for the indicated groups.
Online Figure VI: The expression levels of HGF and VEGF are almost comparable between control and Gab1ECKO mice after HLI. (A) The expression levels of both HGF (upper panel) and VEGF (middle panel) were determined by immunoblotting in the limb muscles after HLI in each group. β-tubulin (bottom panel) was examined for loading control. (B and C) The results were expressed as relative intensity over control sham-operated mice (normalized to β-tubulin level). Values are shown as means ± SEM of three mice. **P<0.01 for the indicated groups. A.U., arbitrary unit(s).
Online Figure VII: The expression level of *TM* mRNA is significantly attenuated in the endothelium of Gab1ECKO mice compared with that of control. CD31-positive ECs were purified from the ischemic limb muscles at 24 h after surgery using MACS system. Total RNAs of the purified ECs of control and Gab1ECKO mice were subjected to real-time RT-PCR analysis. Expression level of *TM* (A) and CypA (B) were analyzed similarly as described in Figure 5 B-E. Values are shown as means ± SEM for three independent experiments. **P<0.01.