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 developmental vascular 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 ($Gab1^{flox/flox}$) mice with $Gab2^{-/-}$ mice to create $Gab1^{flox/flox}$ $Gab2^{-/-}$ 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 (FGF)2. 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.[18,19] 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 speci-

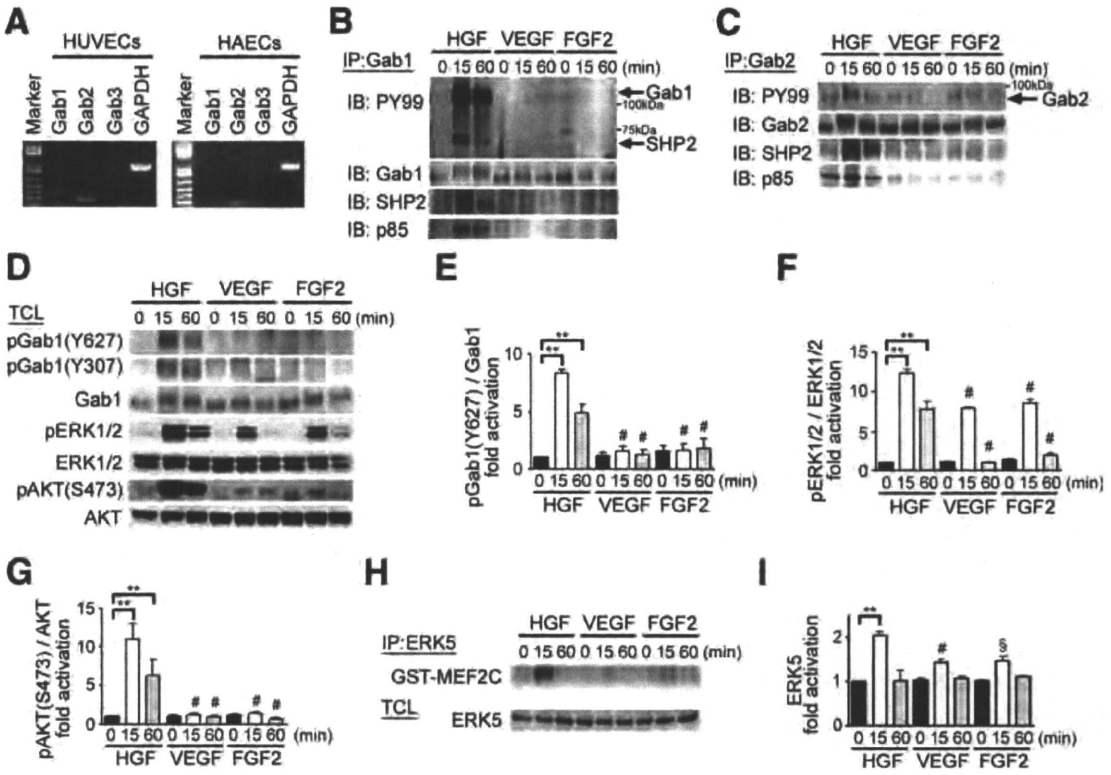

Figure 2. Gab1 and its downstream signaling pathways are strongly activated after stimulation with HGF in HUVECs. A, RT-PCR shows the expression of Gab1 and Gab2 mRNAs, but not Gab3 mRNA, in both HUVECs and human aortic ECs (HAECs). **B and C,** Tyrosine phosphorylation of Gab1 **(B)** and Gab2 **(C)** 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). ^{32}P-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 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; $\#P<0.01$, $\S P<0.05$ vs HGF-treated cells at the same time after stimulation. Values are shown as means±SEM for 3 separate experiments.

ficity 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 (Gab1^{WT}), mutated Gab1 that is unable to bind SHP2 (Gab1$^{\Delta SHP2}$), or mutated Gab1 that is unable to bind p85 (Gab1$^{\Delta p85}$), as described previously.[18,24] We found that Gab1 indeed associated with c-Met after stimulation with HGF in HUVECs overexpressing Gab1^{WT} (Online Figure III). Next, we examined the effect of adenovirus-mediated forced expression of Gab1^{WT}, Gab1$^{\Delta SHP2}$, or Gab1$^{\Delta p85}$ 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 Gab1^{WT} or Gab1$^{\Delta p85}$ compared with control cells expressing β-gal, activation of ERK1/2 was significantly attenuated in HUVECs expressing Gab1$^{\Delta SHP2}$ (Figure 4A and 4B). Furthermore, HGF-induced activation of ERK5 was enhanced in HUVECs expressing Gab1^{WT} compared with control cells expressing β-gal. In addition, enhanced activation of ERK5 was abrogated in HUVECs expressing Gab1$^{\Delta SHP2}$ compared with cells expressing Gab1^{WT} (Figure 4D and 4E). Therefore, the complex formation of Gab1 with SHP2 is required not only for activation of ERK1/2 but also for that of ERK5 after stimulation with HGF in HUVECs.

On the other hand, HGF-induced activation of AKT was significantly enhanced in HUVECs expressing Gab1^{WT} or

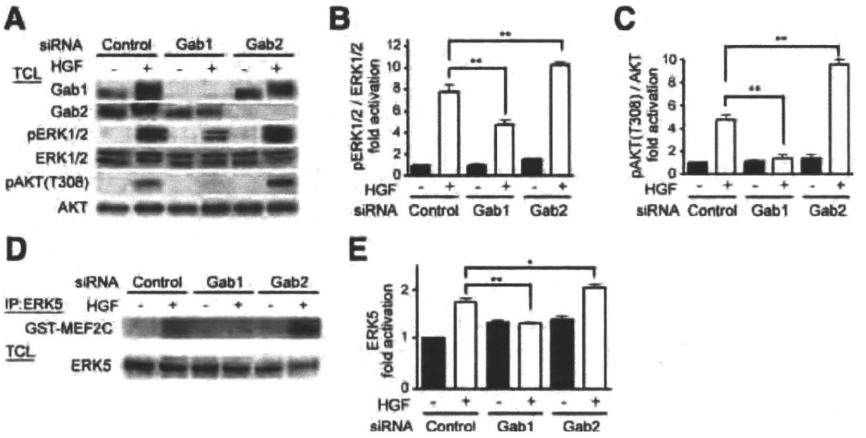

Figure 3. siRNA-mediated knockdown of Gab1, but not Gab2, significantly attenuates activation of ERK1/2, AKT, and ERK5 in response to HGF in HUVECs. A, HUVECs were transfected with control siRNA (Control) or siRNAs targeting either Gab1 or Gab2 for 48 hours. After serum starvation, HUVECs were treated with HGF (20 ng/mL) for 15 minutes. Activation levels of ERK1/2 and AKT were assessed by phospho-specific antibodies. Activation of ERK1/2 and AKT was attenuated in HUVECs transfected with the siRNA targeting Gab1, but enhanced with that targeting Gab2, compared with control cells. **B,** Phosphorylation of ERK1/2 was quantified against total ERK1/2 (n=3). **C,** Phosphorylation of AKT (Ser-473) was quantified 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). ^{32}P-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. $^{*}P<0.05$, $^{**}P<0.01$ for the indicated groups. Values are shown as means±SEM for 3 separate experiments.

Gab1$^{\Delta SHP2}$ compared with control cells, but not in cells expressing Gab1$^{\Delta p85}$ (Figure 4A and 4C). Hence, the complex formation of Gab1 with p85 is critically involved in activation of AKT after stimulation with HGF in HUVECs.

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, Gab1^{WT}, Gab1$^{\Delta SHP2}$, or Gab1$^{\Delta p85}$, and the effect of forced expression of various Gab1 proteins was examined in a monolayer "wound injury" assay. HGF-induced EC migration was significantly enhanced by overexpression of Gab1^{WT}, but significantly repressed by that of Gab1$^{\Delta SHP2}$, compared with control cells expressing β-gal (Figure 4F and 4G). In addition, overexpression of Gab1$^{\Delta p85}$ 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 (MEK5^{DN}), dominant-negative ERK5 (ERK5^{DN}), or dominant-negative MAPK/ERK1 (MEK1^{DN}). HGF-induced endothelial migration was almost abrogated by overexpression of MEK1^{DN}, but not by that of MEK5^{DN} or ERK5^{DN} (Online Figure IV, A and B). In addition, we examined the effect of overexpression of constitutive-active MEK5 (MEK5^{CA}) or constitutive-active MEK1 (MEK1^{CA}) on the cell migration of HUVECs overexpressing Gab1$^{\Delta SHP2}$. Overexpression of MEK1^{CA}, but not MEK5^{CA}, restored cell migration of the HUVECs overexpressing of Gab1$^{\Delta SHP2}$ (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 Gab1^{WT}, but not in those overexpressing either Gab1$^{\Delta SHP2}$ or Gab1$^{\Delta p85}$ (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 Gab1^{WT}, but not that of Gab1$^{\Delta SHP2}$ or Gab1$^{\Delta p85}$, 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 Gab1^{WT}, but not in those overexpressing Gab1$^{\Delta SHP2}$ (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.[16] In addition, Egr1 has also been reported to be critical for ischemia-related gene regulation in the vascular endothelium.[25,26] Thus, we performed further analysis focusing on these 2 genes. To confirm the involvement of Gab1 in the gene regulation of *KLF2* and *Egr1*, we performed siRNA-mediated knockdown of Gab1 in HUVECs. HGF-induced upregulation of both *KLF2* and *Egr1* was abrogated by knockdown of Gab1, but not by that of Gab2 (Figure 5G and 5H). Almost similar result was obtained from immunoblotting analysis for the expression of KLF2 and Egr1 (Figure 5I). KLF2 has been reported to exert antithrombotic and antiinflammatory functions in part through upregulation of the thrombomodulin gene (*TM*).[16,27] Consistently, we confirmed that

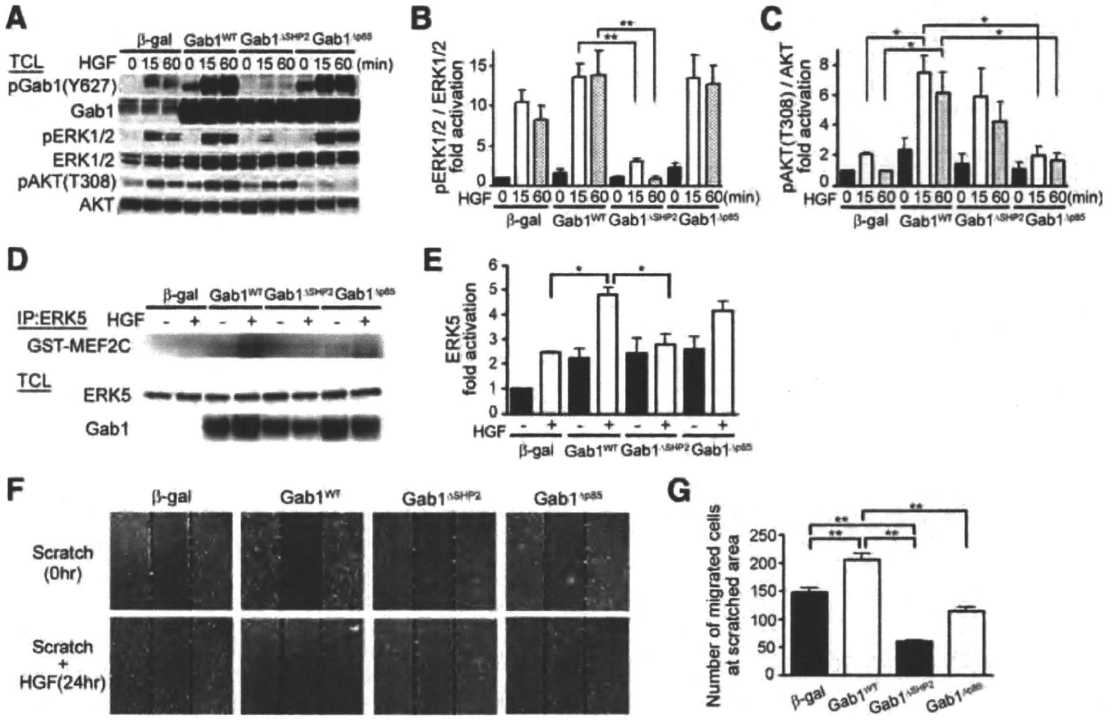

Figure 4. Gab1 is essential for activation of ERK1/2, AKT, and ERK5 and subsequent cell migration after HGF stimulation in HUVECs. A, HUVECs, infected with the indicated adenovirus vectors, were stimulated with HGF (20 ng/mL). Cell lysates were collected and subjected to immunoblotting analyses using the antibodies indicated at the **left**. Expression level of Gab1 was almost comparable in the cells overexpressing Gab1^{WT}, Gab1$^{\Delta SHP2}$, or Gab1$^{\Delta p85}$. Phosphorylation of Gab1 on Tyr-627 was almost abrogated in the cells overexpressing Gab1$^{\Delta SHP2}$. **B,** Phosphorylation of ERK1/2 was quantified against total ERK1/2 (n=3). **C,** Phosphorylation of AKT (Thr308) was quantified against total AKT (n=3). **D,** ERK5 activity was measured by in vitro kinase assay almost similarly described in Figure 2H (n=3). ^{32}P-labeled substrates are shown at the **top** (GST-MEF2C). In parallel, cell lysates were subjected to immunoblotting with anti-ERK5 antibody to confirm the equal amount loading **(middle)** and with anti-Gab1 antibody to verify the overexpression of Gab1 **(bottom). E,** ERK5 activity was quantified and expressed relative to input ERK5 (total cell lysate). The results were expressed as relative intensity over cells expressing β-gal treated with vehicle. **F,** 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 (50 ng/mL) for 24 hours. **G,** Quantification for EC migration in "wound injury" assay. *P<0.05, **P<0.01 for the indicated groups. Values are shown as means±SEM for 3 separate experiments.

TM was indeed upregulated in HUVECs overexpressing Gab1^{WT}, but not in those overexpressing Gab1$^{\Delta SHP2}$ at 4 hours after stimulation with HGF in HUVECs (Online Figure V, A), suggesting that Gab1 might be involved in antithrombotic function through KLF2/TM pathway downstream 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 MEK5^{DN}, ERK5^{DN}, or MEK1^{DN}. HGF-induced upregulation of *KLF2* was almost abrogated by overexpression of either MEK5^{DN} or ERK5^{DN}, but not by that of MEK1^{DN}, 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 MEK1^{DN}, but not by overexpression of either MEK5^{DN} or ERK5^{DN}, 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 (Gab1$^{flox/flox}$), 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 VEGF$_{165}$ (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). Intriguingly, in Gab1ECKO mice, injection of pVAX1-VEGF into ischemic limbs significantly augmented blood flow recovery on day14 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

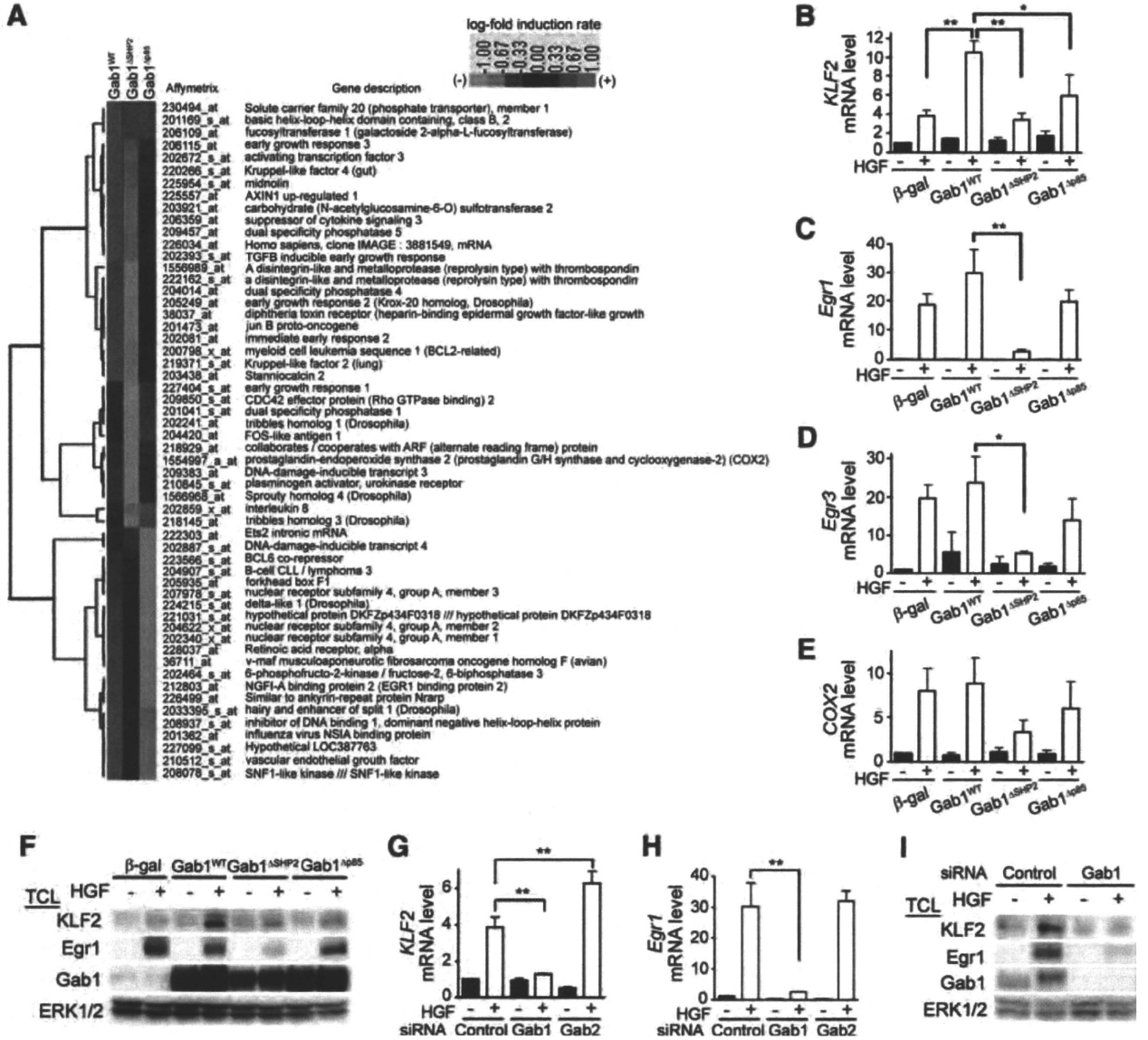

Figure 5. HGF stimulation leads to a distinct pattern of gene expression via Gab1. A, HUVECs infected with the indicated adenovirus vectors were serum-starved and treated with vehicle or 20 ng/mL HGF for 1 hour. Total RNA was purified from the HUVECs and subjected to Affymetrix microarray analysis. Genes corresponding to the criteria described in Methods were subjected to the cluster analysis. **Red and green** represent higher and lower expression than the median for that particular gene, respectively. Color intensity is related to the difference with the median **(black). B through E,** Total RNA was purified from the HUVECs treated with vehicle (−) or 20 ng/mL HGF (+) for 1 hour. The expression levels of *KLF2* **(B),** *Egr1* **(C),** *Egr3* **(D),** and *COX2* **(E)** were analyzed by real-time RT-PCR. **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. **F,** Cell lysates treated with vehicle (−) or HGF (+) for 1 hour were subjected to immunoblotting analyses. **G and H,** HUVECs, transfected with control siRNA (control) or siRNAs targeting either Gab1 or Gab2, were treated with vehicle (−) or HGF (+) for 1 hour. Expression levels of *KLF2* **(G)** and *Egr1* **(H)** mRNAs were analyzed as described for **B through E. I,** Cell lysates treated with vehicle (−) or HGF (+) for 1 hour were subjected to immunoblotting analyses. Values are shown as means±SEM for 3 separate experiments. $^{**}P<0.01$, $^{*}P<0.05$ for the indicated groups.

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

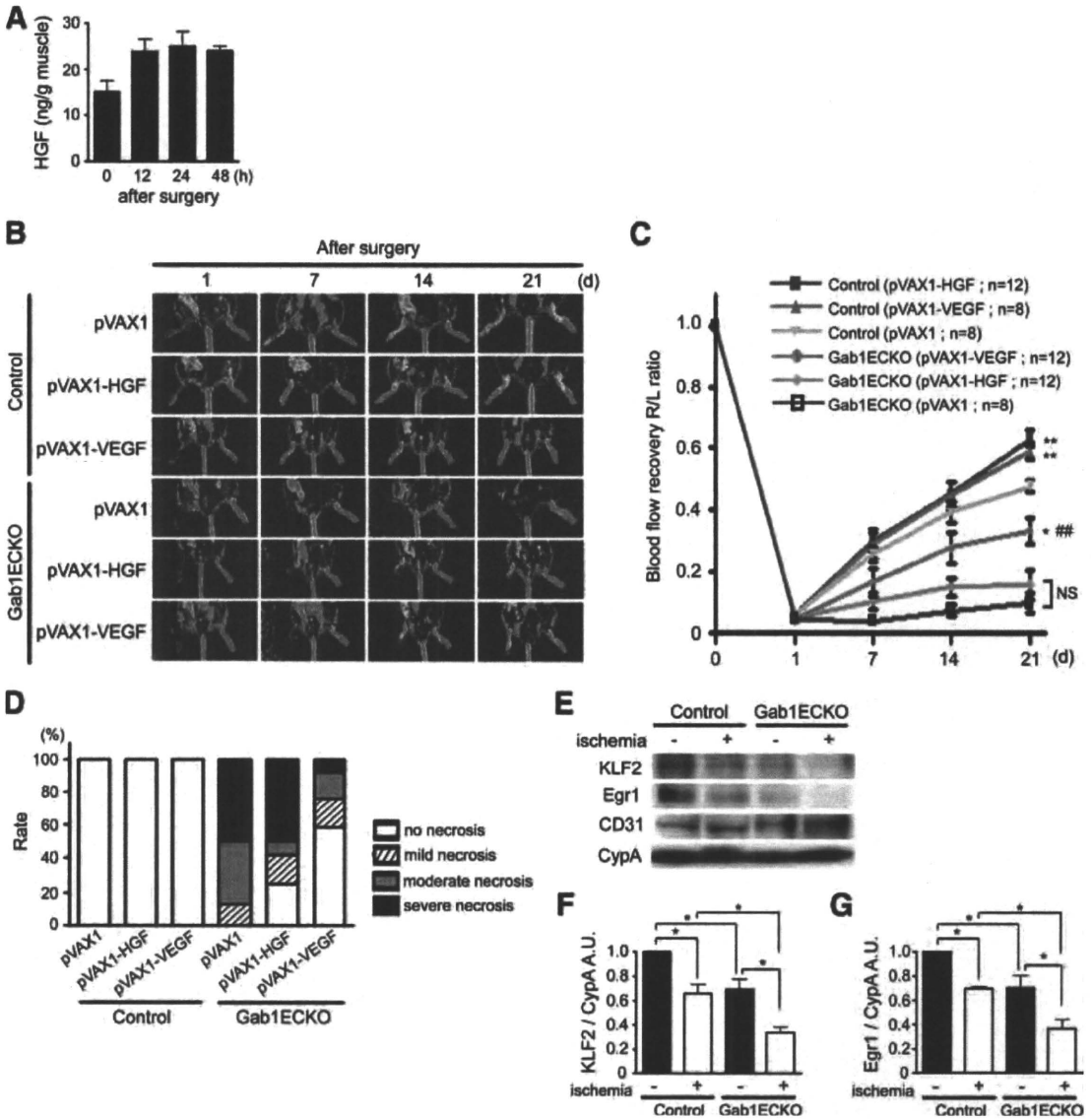

Figure 6. Gene transfer of VEGF, but not HGF, partially ameliorates the limb necrosis after HLI in Gab1ECKO mice. A, Concentration of HGF in the ischemic limb muscle was examined by ELISA (n=3). **B,** Representative LDBF images of mice, injected with control, HGF, and VEGF expression plasmids after HLI on days 1, 7, 14, and 21 after surgery. **Red** represents greater flow; **blue,** less flow. **C,** Quantitative analysis of blood flow recovery after HLI expressed as ischemic **(right)** to nonischemic **(left)** LDBF ratio. Data are from ratio of ischemic right leg vs nonischemic left leg of the mice injected with the plasmid as indicated (n=8 to 12). Values are shown as means±SEM. $^{**}P<0.01$ vs mice injected with pVAX1 in control mice; $\#\#P<0.01$ vs mice injected with pVAX1 in Gab1ECKO; $^{*}P<0.05$ vs mice injected with pVAX1-HGF in Gab1ECKO mice. **D,** Morphometric analysis of the ischemic limb of control and Gab1ECKO mice on day 21 after HLI. Gene transfer of VEGF, but not HGF, partially rescued the necrotic phenotypes of Gab1ECKO mice. **E,** Expression levels of KLF2 and Egr1 were significantly attenuated in the endothelium of Gab1ECKO mice compared with that of control mice both at baseline and on day 1 after HLI. CD31-positive ECs were purified from the limb muscles of control and Gab1ECKO mice using MACS system. Total cell lysates derived from the purified ECs were subjected to immunoblotting analysis. **F and G,** Expression levels of KLF2 and Egr1 were quantified against cyclophilin A (CypA) (n=3). Values are shown as means±SEM for 3 independent experiments. $^{*}P<0.05$. Expression levels of both KLF2 and Egr1 were significantly reduced in Gab1ECKO mice compared with control mice, both before and after ischemia.

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-

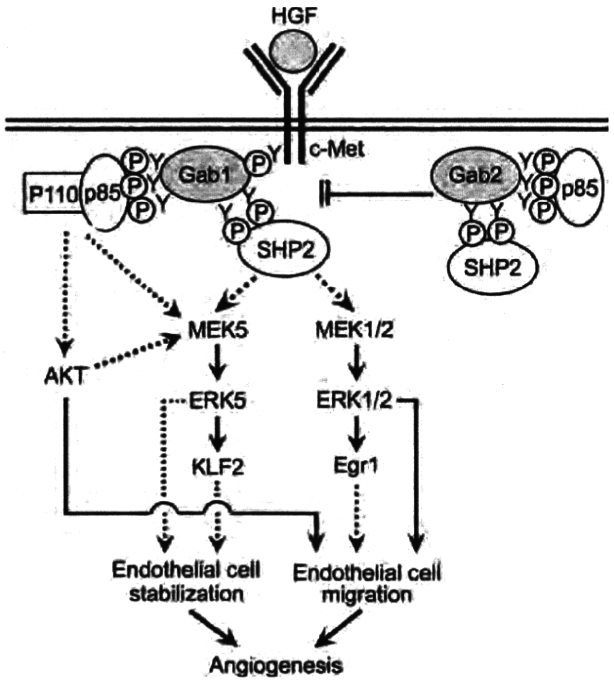

Figure 7. Schematic illustrations of the role of Gab1 in postnatal angiogenesis. HGF induces tyrosine phosphorylation of both Gab1 and Gab2 in the ECs. Both Gab1 and Gab2 associate with SHP2 and p85 on stimulation with HGF. Gab1 is required for activation of ERK1/2, ERK5, and AKT in response to HGF. 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.

mediated knockdown of Gab2 in HUVECs leads to rather 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.[17] KLF2 exerts various vasoprotective, antithrombotic, and antiinflammatory 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.[4,35,36] 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.

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Disclosures

None.

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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 has 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.