

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^{ΔSHP2}, or Gab1^{Δp85}. Phosphorylation of Gab1 on Tyr-627 was almost abrogated in the cells overexpressing Gab1^{ΔSHP2}. **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). ³²P-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^{ΔSHP2} 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^{fllox/fllox}), 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). 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

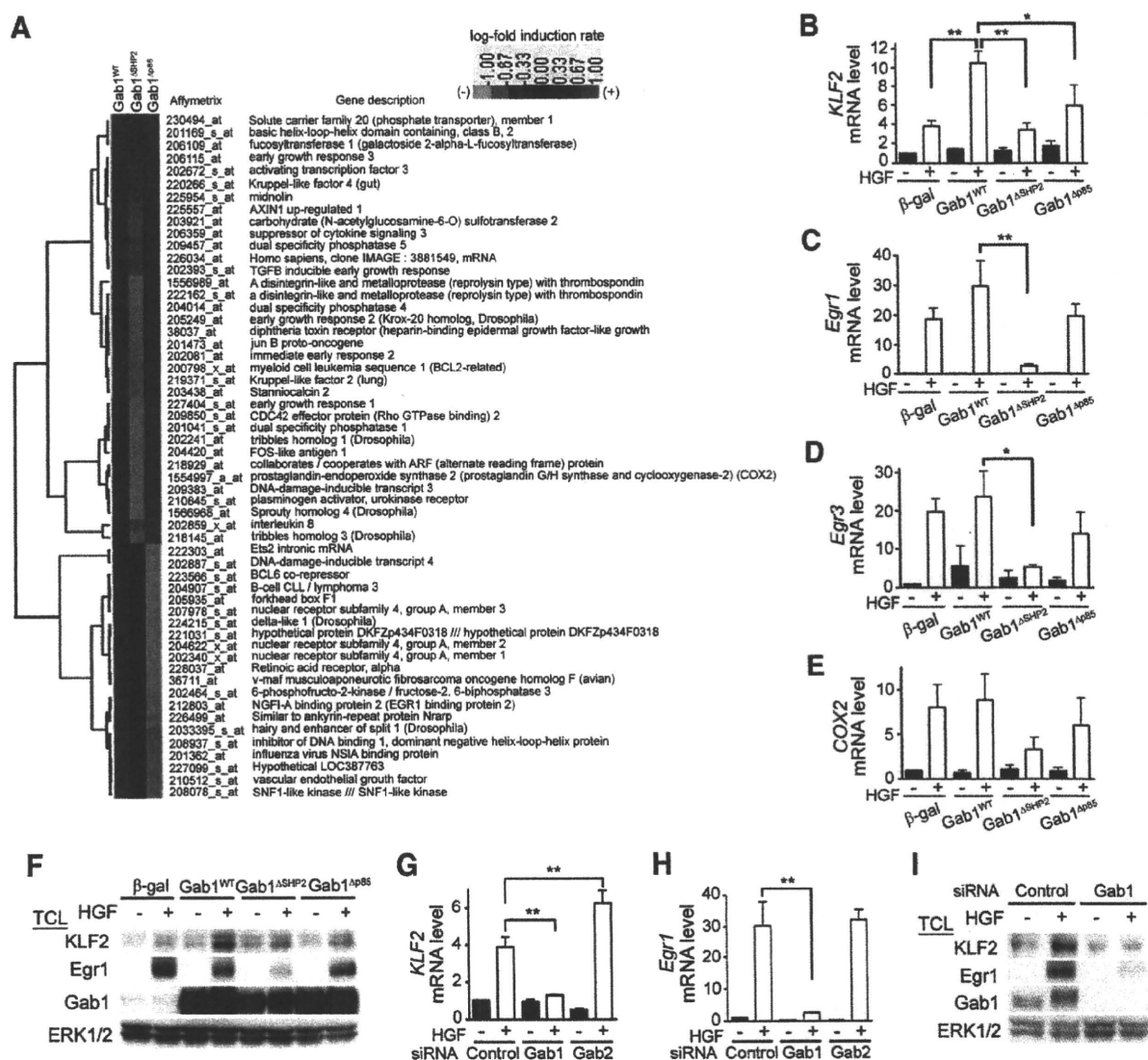


Figure 5. HGF stimulation leads to a distinct pattern of gene expression via Gab1. **A**, HUVECs infected with the indicated adenovirus vectors were serum-starved and treated with vehicle or 20 ng/mL HGF for 1 hour. Total RNA was purified from the HUVECs and subjected to Affymetrix microarray analysis. 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 \pm 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

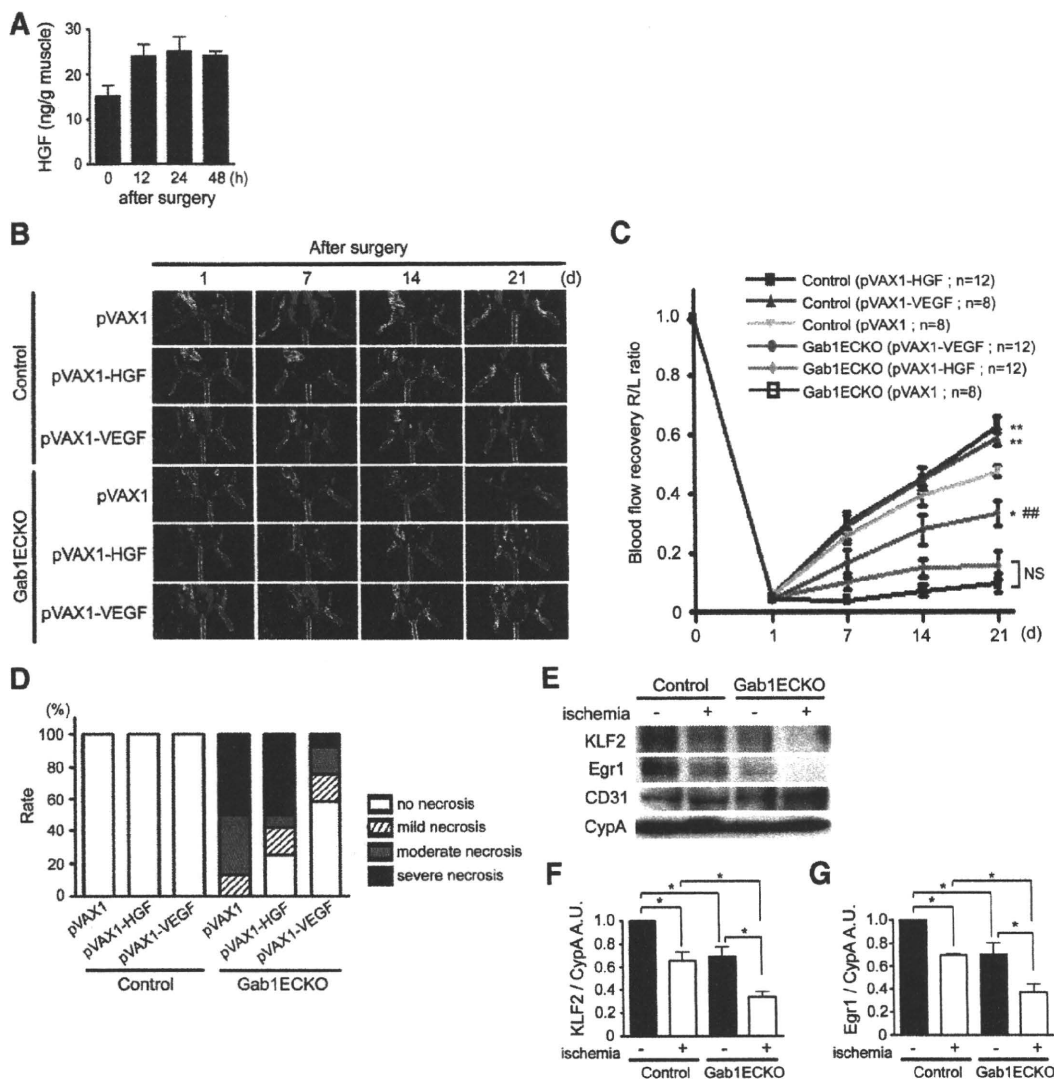


Figure 6. Gene transfer of VEGF, but not HGF, partially ameliorates the limb necrosis after HLI in Gab1ECKO mice. **A**, Concentration of HGF in the ischemic limb muscle was examined by ELISA (n=3). **B**, Representative LDBF images of mice, injected with control, HGF, and VEGF expression plasmids after 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-

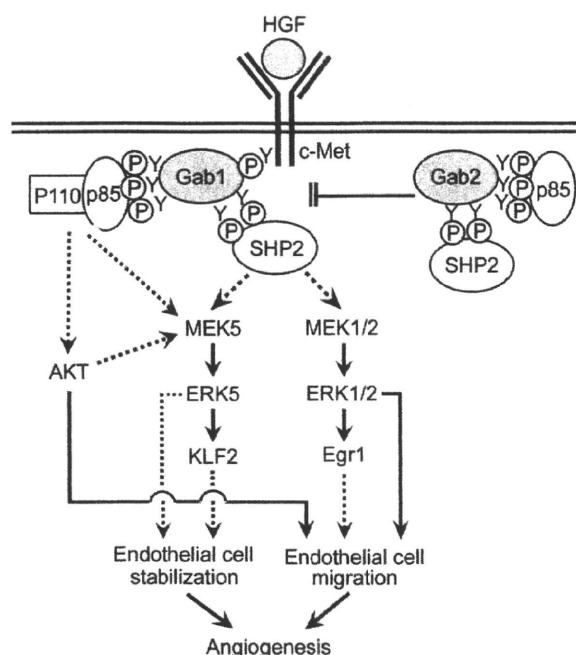


Figure 7. Schematic illustrations of the role of Gab1 in postnatal angiogenesis. HGF induces tyrosine phosphorylation of both Gab1 and Gab2 in the ECs. Both Gab1 and Gab2 associate with SHP2 and p85 on stimulation with HGF. Gab1 is required for activation of ERK1/2, ERK5, and AKT in response to HGF.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

