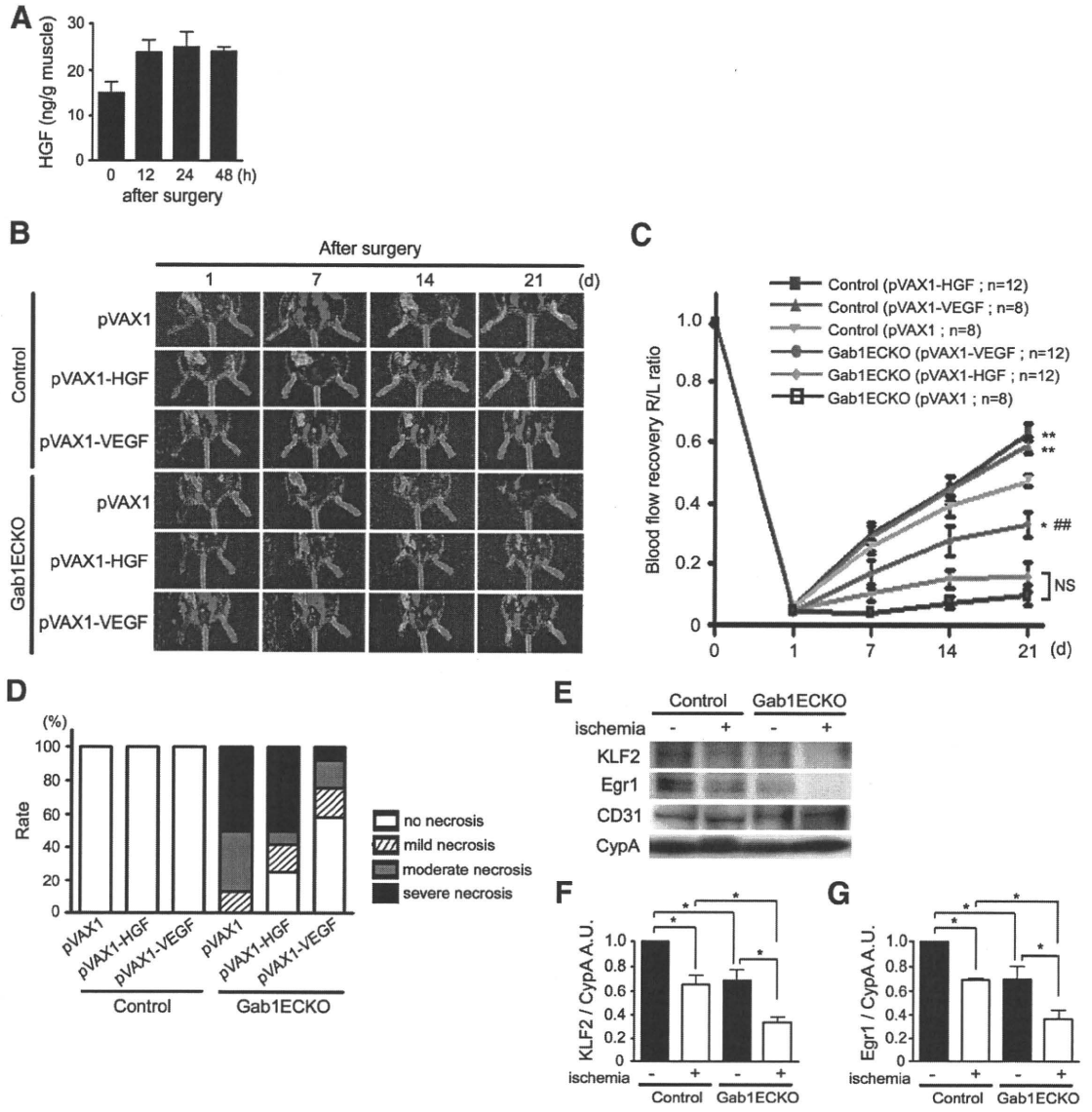

Figure 6. Gene transfer of VEGF, but not HGF, partially ameliorates the limb necrosis after HLI in Gab1ECKO mice. A, Concentration of HGF in the ischemic limb muscle was examined by ELISA (n=3). **B,** Representative LDBF images of mice, injected with control, HGF, and VEGF expression plasmids after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

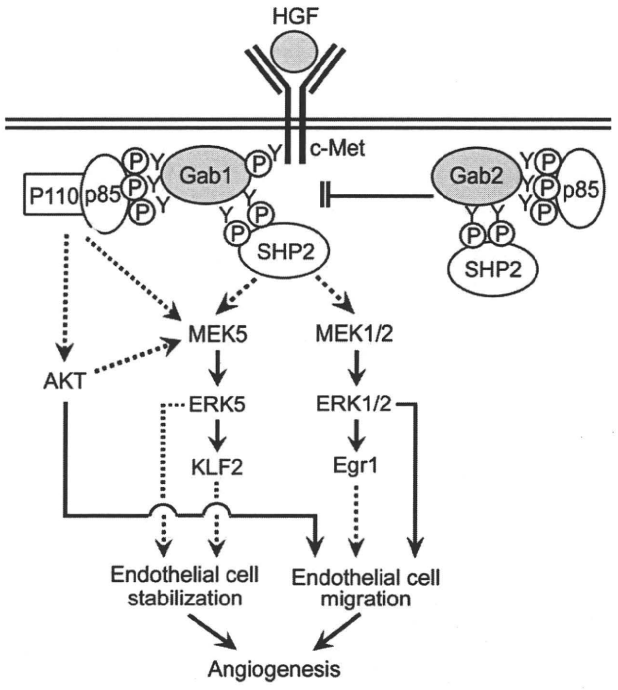

Figure 7. Schematic illustrations of the role of Gab1 in postnatal angiogenesis. HGF induces tyrosine phosphorylation of both Gab1 and Gab2 in the ECs. Both Gab1 and Gab2 associate with SHP2 and p85 on stimulation with HGF. Gab1 is required for activation of ERK1/2, ERK5, and AKT in response to HGF.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

References

1. Gu H, Neel BG. The "Gab" in signal transduction. *Trends Cell Biol.* 2003;13:122–130.
2. Wohrle FU, Daly RJ, Brummer T. Function, regulation and pathological roles of the Gab/DOS docking proteins. *Cell Commun Signal.* 2009;7:22.
3. Itoh M, Yoshida Y, Nishida K, Narimatsu M, Hibi M, Hirano T. Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol Cell Biol.* 2000;20:3695–3704.
4. Sachs M, Brohmann H, Zechner D, Muller T, Hulsken J, Walther I, Schaeper U, Birchmeier C, Birchmeier W. Essential role of Gab1 for signaling by the c-Met receptor in vivo. *J Cell Biol.* 2000;150:1375–1384.
5. Gu H, Saito K, Klaman LD, Shen J, Fleming T, Wang Y, Pratt JC, Lin G, Lim B, Kinet JP, Neel BG. Essential role for Gab2 in the allergic response. *Nature.* 2001;412:186–190.
6. Wada T, Nakashima T, Oliveira-dos-Santos AJ, Gasser J, Hara H, Schett G, Penninger JM. The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. *Nat Med.* 2005;11:394–399.
7. Zhang Y, Diaz-Flores E, Li G, Wang Z, Kang Z, Haviernikova E, Rowe S, Qu CK, Tse W, Shannon KM, Bunting KD. Abnormal hematopoiesis in Gab2 mutant mice. *Blood.* 2007;110:116–124.
8. Seiffert M, Custodio JM, Wolf I, Harkey M, Liu Y, Blattman JN, Greenberg PD, Rohrschneider LR. Gab3-deficient mice exhibit normal development and hematopoiesis and are immunocompetent. *Mol Cell Biol.* 2003;23:2415–2424.
9. Bard-Chapeau EA, Yuan J, Droin N, Long S, Zhang EE, Nguyen TV, Feng GS. Concerted functions of Gab1 and Shp2 in liver regeneration and hepatoprotection. *Mol Cell Biol.* 2006;26:4664–4674.
10. Bard-Chapeau EA, Hevener AL, Long S, Zhang EE, Olefsky JM, Feng GS. Deletion of Gab1 in the liver leads to enhanced glucose tolerance and improved hepatic insulin action. *Nat Med.* 2005;11:567–571.
11. Nakaoka Y, Nishida K, Narimatsu M, Kamiya A, Minami T, Sawa H, Okawa K, Fujio Y, Koyama T, Maeda M, Sone M, Yamasaki S, Arai Y, Koh GY, Kodama T, Hirota H, Otsu K, Hirano T, Mochizuki N. Gab family proteins are essential for postnatal maintenance of cardiac function via neuregulin-1/ErbB signaling. *J Clin Invest.* 2007;117:1771–1781.
12. Weng T, Mao F, Wang Y, Sun Q, Li R, Yang G, Zhang X, Luo J, Feng GS, Yang X. Osteoblastic molecular scaffold Gab1 is required for maintaining bone homeostasis. *J Cell Sci.* 2010;123:682–689.
13. Carmeliet P. Angiogenesis in health and disease. *Nat Med.* 2003;9:653–660.
14. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol.* 2007;8:464–478.
15. Mochizuki N. Vascular integrity mediated by vascular endothelial cadherin and regulated by sphingosine 1-phosphate and angiopoietin-1. *Circ J.* 2009;73:2183–2191.
16. Atkins GB, Jain MK. Role of Kruppel-like transcription factors in endothelial biology. *Circ Res.* 2007;100:1686–1695.
17. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA Jr, Garcia-Cardena G. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J Clin Invest.* 2006;116:49–58.
18. Nakaoka Y, Nishida K, Fujio Y, Izumi M, Terai K, Oshima Y, Sugiyama S, Matsuda S, Koyasu S, Yamauchi-Takihara K, Hirano T, Kawase I, Hirota H. Activation of gp130 transduces hypertrophic signal through interaction of scaffolding/docking protein Gab1 with tyrosine phosphatase SHP2 in cardiomyocytes. *Circ Res.* 2003;93:221–229.
19. Nakaoka Y, Shioyama W, Kunimoto S, Arita Y, Higuchi K, Yamamoto K, Fujio Y, Nishida K, Kuroda T, Hirota H, Yamauchi-Takihara K, Hirano T, Komuro I, Mochizuki N. SHP2 mediates gp130-dependent cardiomyocyte hypertrophy via negative regulation of skeletal alpha-actin gene. *J Mol Cell Cardiol.* 2010;49:157–164.
20. Caron C, Spring K, Laramee M, Chabot C, Cloutier M, Gu H, Royal I. Non-redundant roles of the Gab1 and Gab2 scaffolding adapters in VEGF-mediated signalling, migration, and survival of endothelial cells. *Cell Signal.* 2009;21:943–953.
21. Dance M, Montagner A, Yart A, Masri B, Audigier Y, Perret B, Salles JP, Raynal P. The adaptor protein Gab1 couples the stimulation of vascular endothelial growth factor receptor-2 to the activation of phosphoinositide 3-kinase. *J Biol Chem.* 2006;281:23285–23295.
22. Laramee M, Chabot C, Cloutier M, Stenne R, Holgado-Madruga M, Wong AJ, Royal I. The scaffolding adapter Gab1 mediates vascular endothelial growth factor signaling and is required for endothelial cell migration and capillary formation. *J Biol Chem.* 2007;282:7758–7769.
23. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol.* 2001;230:230–242.
24. Koyama T, Nakaoka Y, Fujio Y, Hirota H, Nishida K, Sugiyama S, Okamoto K, Yamauchi-Takihara K, Yoshimura M, Mochizuki S, Hori M, Hirano T, Mochizuki N. Interaction of scaffolding adaptor protein Gab1 with tyrosine phosphatase SHP2 negatively regulates IGF-I-dependent myogenic differentiation via the ERK1/2 signaling pathway. *J Biol Chem.* 2008;283:24234–24244.
25. Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. *Nat Med.* 2003;9:1026–1032.
26. Khachigian LM. Early growth response-1 in cardiovascular pathobiology. *Circ Res.* 2006;98:186–191.
27. Lin Z, Kumar A, SenBanerjee S, Staniszewski K, Parmar K, Vaughan DE, Gimbrone MA Jr, Balasubramanian V, Garcia-Cardena G, Jain MK. Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. *Circ Res.* 2005;96:e48–e57.
28. Meng S, Chen Z, Munoz-Antonia T, Wu J. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem J.* 2005;391:143–151.
29. Lock LS, Maroun CR, Naujokas MA, Park M. Distinct recruitment and function of Gab1 and Gab2 in Met receptor-mediated epithelial morphogenesis. *Mol Biol Cell.* 2002;13:2132–2146.
30. Maroun CR, Naujokas MA, Holgado-Madruga M, Wong AJ, Park M. The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the met receptor tyrosine kinase. *Mol Cell Biol.* 2000;20:8513–8525.
31. Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, Yang Y, Ulevitch RJ, Lee JD. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. *J Clin Invest.* 2004;113:1138–1148.
32. Regan CP, Li W, Boucher DM, Spatz S, Su MS, Kuida K. Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. *Proc Natl Acad Sci U S A.* 2002;99:9248–9253.
33. Sohn SJ, Sarvis BK, Cado D, Winoto A. ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repressor of vascular endothelial growth factor expression. *J Biol Chem.* 2002;277:43344–43351.
34. Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. *Nature.* 2010;464:1196–1200.

35. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature*. 1995;376:768–771.
36. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature*. 1995;373:699–702.
37. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435–439.
38. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 1996;380:439–442.
39. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66–70.
40. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376:62–66.

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.