

Fig. 5. Apoptotic cardiomyocytes are increased in dominant negative mutant of ATF6 transgenic mice. (a) Electron microscopic analysis. Endoplasmic reticulum were expanded (arrow, 3 and I) and myofilaments were decreased (arrow head, 4 and II) in cardiomyocytes of dnTg mice. Degenerated cardiomyocytes were observed in dnTg mice (5 and III). Scale bar, 1 μ m. WT, wild-type littermates. (b) TUNEL-positive cardiomyocytes (brown) in hearts of WT or dnTg mice. Scale bar, 10 μ m. The graph showed quantitative analysis for TUNEL-positive cardiomyocytes at 8 weeks of age. * $P < 0.05$ versus WT. $n = 5$. Scale bar, 10 μ m. (c) Expression levels of CHOP and PUMA in hearts of WT or dnTg mice were quantified by RT-PCR analysis, normalized against GAPDH mRNA expression. * $P < 0.05$ versus WT. $n = 4$. (d) Left ventricular end-diastolic dimension (LVDd) and fractional shortening (FS) were examined at 8 weeks of age by echocardiogram. Scale bar, 2 mm. * $P < 0.01$ versus WT Bcl-2(-) mice, † $P < 0.05$ versus dnTg Bcl-2(-) mice.

mice than in wild-type littermates even when they were treated with AEBFS (Supplemental Fig. 3a). On the other hand, the survival rate was lower in dnTg mice than in wild-type littermates (Supplemental Fig. 3b). These results suggest that activation of ATF6 has protective effects on cardiomyocytes and prevents cardiac remodeling after MI.

4. Discussion

Recent reports show that the ER stress response is activated in the heart exposed to prolonged stresses such as pressure overload [14] as well as acute stresses such as ischemia/reperfusion [26]. Although

there is a report showing that BiP expression is detected in cardiomyocytes of border zone after MI [27], expressions of other ER stress-related factors are not known in hearts after MI. We revealed that all three branches of ER stress sensor, such as ATF6, PERK, and IRE1, were activated in the MI heart (Fig. 1, Supplemental Fig. 1). The mRNA and protein levels of an ER chaperone BiP, which is regulated by ATF6, were also increased (Fig. 1). Furthermore, the mRNA levels of CHOP were increased, which has been reported to induce apoptosis under excessive ER stress (Fig. 1b). These results suggest that the ER stress response is induced in the heart by MI and that excessive ER stress induces apoptosis of cardiomyocytes after MI.

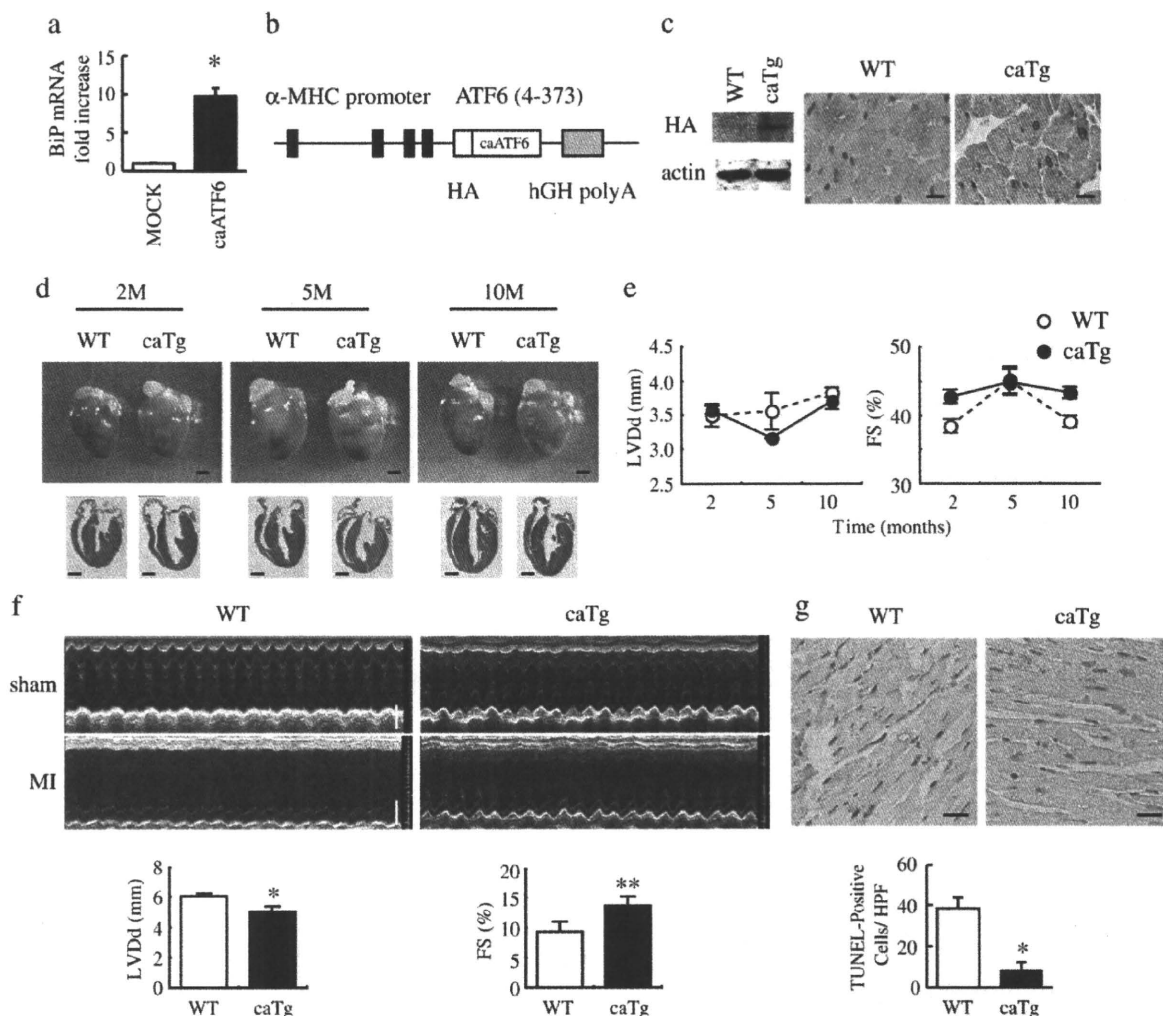


Fig. 6. The constitutively active mutant of ATF6 transgenic mice. (a) Expression levels of BiP in HEK293 cells transfected with MOCK or an expression plasmid encoding the constitutively active form of ATF6 were quantified by RT-PCR analysis, normalized against GAPDH mRNA expression. * $P < 0.05$ versus MOCK. (b) Schematic diagram represented the constitutively active mutant of ATF6 (caATF6) transgene. HA-tagged caATF6 was subcloned between the murine α -MHC promoter and human growth hormone (hGH) polyA. (c) Western blot analysis and immunohistochemical staining (brown) for HA in hearts of wild-type littermates (WT) or caTg mice at 2 months of age. Scale bar, 10 μ m. (d) Gross morphology (upper images) and sections (lower images) of WT or caTg murine hearts at 2, 5 and 10 months (M) of age. Scale bar, 1 mm. (e) Left ventricular end-diastolic dimension (LVDD) and fractional shortening (FS) were examined at 2, 5 and 10 months of age by echocardiogram. (f) Left ventricular end-diastolic dimension (LVDD) and fractional shortening (FS) were examined at 14 days after myocardial infarction by echocardiogram. Scale bar, 2 mm. * $P < 0.05$, ** $P < 0.01$ versus WT. (g) TUNEL-positive cells (brown) in hearts at 24 h after MI. Scale bar, 10 μ m. The graph showed quantitative analysis for TUNEL-positive cells at 24 h after MI. Scale bar, 100 μ m. * $P < 0.05$ versus vehicle. $n = 3$.

ATF6 is a major transcription factor to induce gene expression of ER chaperones as well as ERAD components [22]. We firstly produced MI in wild-type mice in the presence of an ATF6 inhibitor, AEBFSF. AEBFSF has been reported to inhibit site-1 protease in vitro, which cleaves and activates ATF6 [19]. In this study, AEBFSF inhibited an increase of activated forms of ATF6 (p50ATF6) after MI (Fig. 2a), suggesting that AEBFSF indeed suppressed ATF6 function also in vivo. AEBFSF reduced survival rate and worsened cardiac function after MI (Fig. 2b and c, Supplemental Table 1). Although it remains unclear how inhibition of ATF6 function deteriorated cardiac remodeling after MI, suppression of BiP might be one reason (Fig. 3a). BiP is a chaperone to reduce the ER stress, and a recent report shows that BiP has protective effects on cardiomyocytes against ischemic injury [28]. On the other hand, BiP null mice exhibit embryonic lethality because of an increase of apoptosis in the inner cell mass [29]. Suppression of BiP by AEBFSF might increase apoptosis in the ischemic heart due to excessive ER stress, resulting in deterioration of cardiac remodeling. Indeed, the number of TUNEL-positive cells and expression level of

CHOP were increased more in the MI heart by AEBFSF treatment (Fig. 3b and c). The in vitro result that the number of CoCl₂-induced apoptotic cardiomyocytes was increased more by AEBFSF further supports this idea (Fig. 3day).

Targeted disruption of XBP1 gene is embryonic lethal in mice because of cardiac developmental defects [30]. Double knockout mice of ATF6 α and ATF6 β gene are also embryonic lethal [22]. On the other hand, PERK knockout mice exhibit death of pancreatic β cells and develop diabetes [12]. Although these studies suggest that the ER stress response is important for development and homeostasis of organs, the physiological role of the ER stress response in the heart has not been fully elucidated. To clarify the role of the ER stress response, especially ATF6 in the heart under physiological state, we established mice that expressed dominant negative mutant of ATF6 in the heart (Fig. 4a). Heart size was enlarged and cardiac function was impaired in dnTg mice even without pathological stresses (Fig. 4f and g, Supplemental Table 2), suggesting that ATF6 is necessary to maintain cardiac structure and function in adulthood.

Electron microscopic analysis showed that endoplasmic reticulum was expanded in cardiomyocytes of dnTg mice (Fig. 5a), suggesting that the ER stress is increased. The number of TUNEL-positive cardiomyocytes was increased (Fig. 5b) and mRNA levels of ER stress-induced apoptosis-related genes such as CHOP and PUMA were upregulated in the heart of dnTg mice (Fig. 5c). Impaired ER stress response by dominant negative mutant of ATF6 might increase apoptotic cardiomyocytes even under physiological state, resulting in cardiac dysfunction. This idea was supported by the result that overexpression of Bcl-2 significantly improved cardiac function in dnTg mice (Fig. 5d, Supplemental Table 3).

Cardiac remodeling after MI was prevented in caTg mice (Fig. 6f, Supplemental Table 5), and was deteriorated in dnTg mice (Supplemental Fig. 2b). Furthermore, the reduced survival rate with AEBSF was improved in caTg mice (Supplemental Fig. 2a). These results suggest that ATF6 activation after MI is protective in the heart, which is consistent with the recent result of ischemia/reperfusion injury in hearts [26]. Although the decrease of TUNEL-positive cells might be involved in the prevention of cardiac remodeling after MI (Fig. 6g), precise mechanisms of how overexpression of ATF6 ameliorates cardiac remodeling remain to be determined.

This study indicates that activation of the ER stress response factor ATF6 plays a critical role in protecting hearts under the pathological state and maintaining cardiac function under the physiological state.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2010.03.020.

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ORIGINAL ARTICLE

SNPs on chromosome 5p15.3 associated with myocardial infarction in Japanese population

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Myocardial infarction (MI) occurs as the result of complex interactions of multiple genetic and environmental factors. By conducting a genome wide association study in a Japanese population using 210 785 single nucleotide polymorphism (SNP) markers, we identified a novel susceptible locus for MI on chromosome 5p15.3. An SNP (rs11748327) in this locus showed significant association in several independent cohorts (combined $P=5.3 \times 10^{-13}$, odds ratio=0.80, comparison of allele frequency). Association study using tag SNPs in the same linkage disequilibrium block revealed that two additional SNPs (rs490556 and rs521660) conferred risk of MI. These findings indicate that the SNPs on chromosome 5p15.3 are novel protective genetic factors against MI.

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Keywords: association; genome; myocardial infarction; SNP

INTRODUCTION

Myocardial infarction (MI) leads to principal cause of death in developed countries. MI is characterized by the rapid development of coronary thrombosis following atherosclerotic plaque instability,¹ which leads to necrosis of myocardium and might result in sudden death. Despite the change in lifestyle and recent development of biomarkers, pharmacological intervention and percutaneous coronary intervention using drug eluting stents, the mortality is still high.

We started genome wide association studies (GWAS) of this disorder using nearly 90 000 gene-based single nucleotide polymorphisms (SNPs) (<http://snp.ims.u-tokyo.ac.jp/>)² by high-throughput multiplex-PCR invader assay system,³ and identified several genes conferring risk of MI including *LTA*.^{4–6} Although the roles of these susceptible genes in MI pathogenesis are under investigation, these findings showed the potent power of GWAS, which is hypothesis free, to identify unexpected anchors to further understand the disease. Through examining the LTA cascade by combination of biological and genetic analyses, we have identified additional MI susceptible genes.^{7–9}

Genetic variants that confer susceptibility to MI have been indicated to be present on several chromosomal loci.^{10–17} These studies, however, were conducted in individuals from European descent. Therefore, we carried out a systematic GWAS using 210 785 SNPs for MI in Japanese population. We report here identification of SNPs on chromosome 5p15.3 as a novel protective genetic factor against MI. We also examined Taiwanese population to see its universality in another population.

MATERIALS AND METHODS

DNA samples

For the genome wide association study and subsequent second-stage screening, MI case and control subjects (mixed cases with other diseases including asthma, breast cancer, lung cancer, hyperthyroidism, osteoporosis, chronic obstructive pulmonary disease, pollinosis and atopic dermatitis) were obtained from the BioBank Japan project (<http://biobank.jp.org/>). The characteristics of the third, fourth cohorts and the diagnosis of define MI has been described previously.^{8,9} For Taiwanese population, subjects were recruited from the Kaohsiung Medical University Hospital, Taiwan.⁹ All Taiwanese subjects are of Chinese descent. All

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study subjects provided written informed consent to participation in this study, or if they were under 20 years old, their parents gave consent. Characteristics of the study subjects were summarized in Supplementary Table 1. The protocol was approved by the Ethical Committee at the Center for Genomic Medicine, The institute of physical and chemical research (RIKEN), Yokohama and of each participating institution and by the Internal Review Board of the Kaohsiung Medical University Hospital, Kaohsiung.

SNP genotyping

The genotyping methods for GWAS and second-stage screening were described previously.¹⁸ For third- and fourth-stage screening, we used multiplex-PCR invader assay described previously.³ In the Taiwanese population, the SNPs were genotyped using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Haplotype block and haplotype frequency were estimated using Haploview v4.0.¹⁹ We used this software to select tag SNPs with a pairwise tagging mode and applied a permutation test for haplotype analysis. We also applied haplotype analysis using the program THESIAS²⁰ and conditional log-likelihood with Akaike information criterion (AIC): $AIC = -2 \times (\text{the maximized value of the conditional log-likelihood}) + 2 \times (\text{the number of parameters})$. As the number of parameters, we used the number of alleles/haplotypes with frequencies >0.01 that were used for each model. In the logistic regression analysis of an SNP, we first applied a one degree of freedom (1 d.f.) likelihood ratio test to determine whether a 1-d.f. multiplicative allelic effects model of a 2-d.f. full genotype model was more appropriate.²⁰ As we found no significant difference from the full genotype model ($P > 0.05$), we assumed a multiplicative allelic effects mode. Next, we performed a forward logistic regression analysis, where we started by assessing whether the most significant SNP was sufficient to model the association among the SNP set. For this, we used a 1-d.f. likelihood ratio test for adding each of the remaining SNPs to the model by assuming multiplicative allelic effects for the additional SNPs. Relationship between patients' clinical profile and genotype information were assessed by one-way analysis of variance and χ^2 -test.

Northern blot analysis

Human multiple-tissue northern blots I, II (Clontech, Palo Alto, CA, USA) or First choice northern blot (Ambion, Austin, TX, USA) 1, 2 were pre-hybridized and hybridized with α -[³²P]-dCTP-labeled genomic fragments prepared by PCR using 106 primer pairs as probes (Primer pairs are listed in Supplementary Table 2). Washed membranes were exposed to bioimaging plate for 4–6 h. We detected signal with bioimaging analyzer (FLA7000, FUJIFILM, Tokyo, Japan) according to the manufacturer's instructions.

RESULTS

Genome wide association analysis

We performed staged GWAS that include three screening stages as shown in Figure 1. To avoid false-negative results, we set a very loose threshold in the first-stage screening. We first genotyped 268 068 SNPs with 194 MI cases and 1539 controls (first set of each MI and control) enrolled in BioBank Japan. We successfully obtained genotype information at 210 785 SNP loci. The genomic inflation factor (λ) was 1.03 on the basis of the P -values from the Cochran-Armitage trend test, indicating there is no population stratification. We then selected 8740 SNPs showing P -values <0.02 for the second-stage screening, and genotyped the second set of 1394 MI patients and 1425 control individuals (Supplementary Table 3). Distribution of P -values for these SNPs were summarized in Supplementary Table 4. We also assessed population stratification in second-stage samples by comparing with HapMap samples using principal component analyses,²¹ and found that these samples did not show any sign of population stratification (Supplementary Figure 1). After the second-stage screening, we identified two SNPs showing statistical significance after

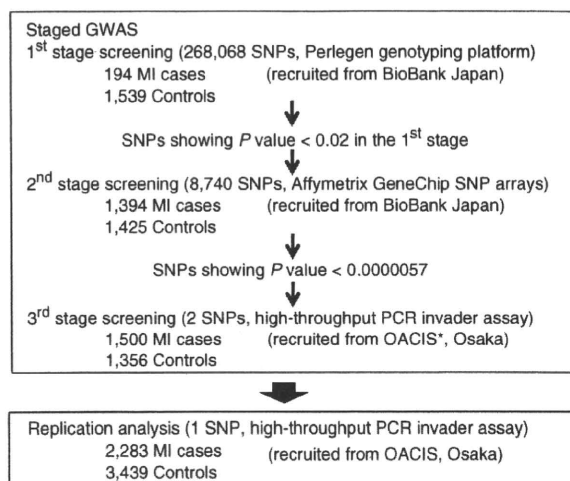


Figure 1 Study design for the GWAS. *Osaka Acute Coronary Insufficiency Study group.

Bonferroni's correction (cutoff P -value <0.0000057). One SNP (rs3782886) was located within *BRAP* on chromosome 12q24 (Supplementary Table 3), previously reported to be associated with susceptibility to MI in two Asian populations.⁹ The remaining SNP (rs11748327) on chromosome 5p15.3 showed P -value of 1.8×10^{-6} in second-stage screening, and was verified by genotyping the third-stage samples (1500 cases and 1356 controls). Subsequent joint analyses for the associations of three panels showed the P -value with genome wide significance (combined $P = 1.4 \times 10^{-9}$, odds ratio = 0.77; Table 1). This association was further verified by replication panels with 2283 cases and 3439 controls (Table 1). Combined analysis of the four panels using Mantel-Haenszel test showed strong association of the SNP and MI, with a χ^2 value of 56.0 ($P = 5.3 \times 10^{-13}$; comparison of allele frequency) and odds ratio was 0.80 (95% confidence interval: 0.75–0.85; Table 1).

Linkage disequilibrium and haplotype analysis

The marker SNP (rs11748327) was located within ~ 250 kb linkage disequilibrium (LD) block constructed on the basis of HapMap JPT data (<http://www.hapmap.org>)²² using Haploview software¹⁹ (Figure 2). To examine whether other genetic variation(s) in this block is associated with MI, we selected 15 tag SNPs in addition to rs11748327 from SNPs to have minor allele frequency $>5\%$ with pairwise tagging and r^2 threshold of 0.8. We compared allelic frequency of these SNPs in the third panel of each MI and control and found that two additional SNPs (rs490556 and rs521660) were significantly associated with MI after Bonferroni's correction (Table 2).

The two SNPs, rs490556 and rs521660, were in LD to the marker SNP rs11748327 with r^2 of 0.59 and 0.79, respectively (Table 2). Then, we further genotyped the replication panel of 2283 cases and 3439 controls for the two SNPs and found again significant association between these SNPs and MI (Table 3). Two of the haplotypes based on these SNPs showed significant association with MI (Table 4). Therefore, we further examined the effect of these haplotypes by THESIAS²⁰ and observed a significant effect on disease susceptibility between the most and second-most frequent haplotypes that could be distinguished by rs490556. Considering the conditional log-likelihoods with AIC, indicating that rs490556 revealed smaller AIC value than the haplotype model, we assumed that rs490556 alone rather than the

Table 1 Association of rs11748327 SNP with MI

Stage	Panel	MI						Controls						Comparison of allele frequency					
		11	%	12	%	22	%	SUM	11	%	12	%	22	%	SUM	χ^2	P-value	OR	95% CI
Staged GWAS	1st	130	67.0	52	26.8	12	6.2	194	866	56.3	575	37.4	97	6.3	1538	5.5	1.9×10^{-2}	0.73	0.56–0.95
	2nd	896	64.4	436	31.3	60	4.3	1392	758	55.3	531	38.7	82	6.0	1371	22.8	1.8×10^{-6}	0.74	0.83–0.65
	3rd	938	62.8	480	32.1	76	5.1	1494	770	57.1	490	36.4	88	6.5	1348	10.2	1.4×10^{-3}	0.82	0.72–0.93
	Combined															40.7	$1.4 \times 10^{-9*}$	0.77	0.71–0.84
Validation	Replication	1437	62.9	740	32.4	106	4.6	2283	1964	57.5	1254	36.7	197	5.8	3415	16.7	4.4×10^{-5}	0.83	0.76–0.91
	Combined															56.0	$5.3 \times 10^{-13*}$	0.80	0.75–0.85

Abbreviations: CI, confidence interval; GWAS, genome wide association studies; MI, myocardial infarction; OR, odds ratio; SNP, single nucleotide polymorphism.
*P-value was calculated by Mantel–Haenszel test.

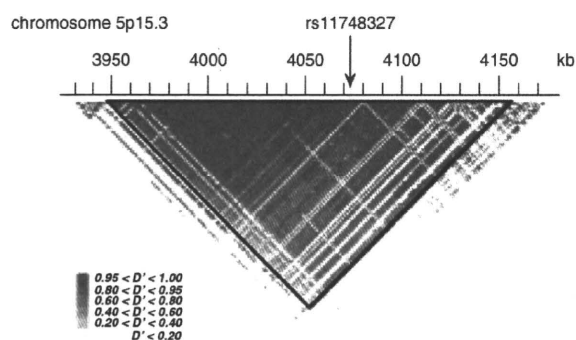


Figure 2 LD (D') block containing the marker SNP (rs11748327) on chromosome 5p15.3.

haplotypes well explained an association with MI. We also applied a logistic regression analysis to search for combinatorial effects of other SNPs to rs490556, but failed to find them. These results indicated that rs490556 itself or other SNPs with high LD to rs490556 are genetically associated with MI.

We also examined the possibility of confounding effect by age, sex and classical risk factors including diabetes, hypertension, smoking, hyperlipidemia within patients group using one-way analysis of variance and χ^2 test, and found no relation between genotype and these factors (data not shown), indicating that the significant SNPs are an independent risk factor of MI.

We further conducted the association between the three tag SNPs and MI with ~550 cases and 800 controls from a Taiwanese population. However, the results were not consistent with the association for MI in Japanese population (Supplementary Table 5).

Gene discovery at 5p15.3 locus

National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>) contained only one expressed sequence tag, DA489076.1, in the genomic region within the LD block. Therefore, we examined expression of DA489076.1 in cDNA derived from 13 human tissues including heart, lung, liver, skeletal muscle, placenta, peripheral blood leukocyte, lymph node, adipose, aorta, brain, fetal brain, coronary artery smooth muscle and coronary artery endothelial cells. However, the expression was not detectable in all tissues examined (data not shown). To explore whether other unidentified transcripts are present in this genomic region, we examined mRNA expression in human adult tissues by northern blot analyses using 106 amplicons as probes (Primer pairs are listed in Supplementary Table 2) that cover the entire genomic region of the block except for repetitive sequences. We

could not find obvious signal in all tissues examined (data not shown). Although we also examined micro-RNA (miRNA) and copy number variation databases (<http://www.mirbase.org/search.shtml> and https://gwas.lifesciencedb.jp/cgi-bin/cnvdb/cnv_top.cgi, respectively), we were not able to obtain any information for the genomic region.

DISCUSSION

Through a GWAS in a Japanese population using 210 785 SNP markers, we identified SNPs on chromosome 5p15.3 as a novel protective genetic factor against MI. The association that we observed in the Japanese population could not be replicated in the Taiwanese population. This might be due to a lack of the power ($1-\beta$; 0.17 in comparison of allele frequencies for these SNPs) or genetic difference between the two populations. We also cannot exclude the possibility that other variants in this genomic region confer risk of MI in the Taiwanese population. The loci on chromosome 5p15.3 and *BRAP* were not detected in the previous GWAS from Europe and the United States; this failure may be due to the difference among ethnicity in allelic frequencies, which affects power of the study and also the effect size. Other reasons might include the ethnic difference in the precise LD pattern, possibility of unidentified hidden SNPs for Caucasian decent, various biases such as publication bias, leaving open the question of association in other populations for these loci.

We could not find replicated previous results for *LTA* and *PSMA6* in the first-stage screening. The estimated powers of the first-stage screening to replicate positive association for *LTA* and *PSMA6* were 0.28 and 0.24, respectively. The significant SNP in *LGALS2* was not on the SNP list of Perlegen genotyping system. Therefore, we think one of the reasons might be lack of the power of this study. Biases including publication bias, sampling bias, cannot be excluded. For chromosome 9p21 locus, the *P*-value in the first-stage screening was 0.0018 (rs1412834). At the second stage, it was 0.0098 and did not pass the threshold. As our aim did not include replication of the previous findings, threshold *P*-value at each screening stage was not appropriate for replication study.

In the genomic region of the LD block on chromosome 5p15.3, we were not able to detect any transcript by our analyses. It is possible we cannot detect unidentified some small non-coding RNAs <100 base pairs, particularly miRNAs. miRNA has important functions in gene regulation in animals and plants by binding to target sites in the 3' untranslated regions on mRNAs of protein-coding genes to direct their posttranscriptional repression.²³ In fact, recent studies indicated that a single substitution in the match of the miRNA seed to its target site can abolish gene repression.²⁴ SNPs in miRNA including pri-miRNAs, pre-miRNAs and mature miRNA could influence the processing and/or target selection of miRNAs and affect

Table 2 Association analysis of the 15 tag SNPs with MI

dbSNP ID	MI							Controls							P-value ^a	r ² with rs11748327
	11	%	12	%	22	%	SUM	11	%	12	%	22	%	SUM		
rs511664 C>T	1232	86.0	193	13.5	8	0.6	1433	1150	88.3	143	11.0	9	0.7	1302	1.6×10 ⁻¹	0.03
rs505800 A>G	763	51.4	599	40.3	123	8.3	1485	666	49.6	551	41.1	125	9.3	1342	4.0×10 ⁻¹	0.3
rs2008927 C>T	700	47.3	614	41.5	166	11.2	1480	650	49.2	524	39.6	148	11.2	1322	7.2×10 ⁻¹	0.18
rs631942 G>A	795	53.9	558	37.8	122	8.3	1475	660	48.9	569	42.1	121	9.0	1350	2.9×10 ⁻¹	0.4
rs10060583 C>T	566	38.1	686	46.2	233	15.7	1485	547	40.9	611	45.6	181	13.5	1339	9.1×10 ⁻¹	0.12
rs490556 T>C	863	58.1	521	35.1	102	6.9	1486	681	50.7	549	40.9	113	8.4	1343	2.4×10 ⁻³	0.59
rs521660 G>A	762	55.3	515	37.3	102	7.4	1379	627	49.1	530	41.5	120	9.4	1277	1.5×10 ⁻²	0.79
rs1187466 T>A	630	44.3	630	44.3	162	11.4	1422	579	46.9	540	43.8	115	9.3	1234	1.1×10 ⁻¹	0.11
rs1187463 C>A	743	50.4	600	40.7	130	8.8	1473	607	46.0	563	42.7	150	11.4	1320	7.5×10 ⁻²	0.65
rs903083 C>T	1334	89.4	156	10.5	2	0.1	1492	1180	88.0	155	11.6	6	0.4	1341	2.6×10 ⁻¹	0.02
rs10512709 G>A	607	41.3	682	46.5	179	12.2	1468	559	42.3	611	46.3	151	11.4	1321	8.0×10 ⁻¹	0.17
rs1187477 C>T	1169	77.9	308	20.5	23	1.5	1500	1025	75.6	311	22.9	20	1.5	1356	3.0×10 ⁻¹	0.04
rs1209069 C>T	816	58.6	496	35.6	81	5.8	1393	779	61.2	435	34.2	58	4.6	1272	1.4×10 ⁻¹	0.04
rs1493470 G>A	827	55.9	566	38.3	86	5.8	1479	729	54.5	504	37.7	104	7.8	1337	2.4×10 ⁻¹	0.78
rs1187483 T>C	578	39.6	663	45.4	220	15.1	1461	478	36.0	635	47.8	215	16.2	1328	1.2×10 ⁻¹	0.41

Abbreviations: MI, myocardial infarction; SNP, single nucleotide polymorphism.
^aComparison of allelic frequency and adjusted for Bonferroni's correction.

Table 3 Association of the rs490556 and rs521660 with MI

dbSNP ID	Samples	Cases							Controls							Comparison of allele frequency			
		11	%	12	%	22	%	Total	11	%	12	%	22	%	Total	χ ²	P-value	OR	95% CI
rs490556 T>C	3rd	863	58.1	521	35.1	102	6.9	1486	681	50.7	549	40.879	113	8.4	1343	14.4	1.5×10 ⁻⁴	0.79	0.70–0.89
	Replication	1324	58.4	802	35.4	141	6.2	2267	1786	51.9	1399	40.68	254	7.4	3439	20.6	5.7×10 ⁻⁶	0.82	0.75–0.89
	Combined															33.8	4.0×10 ^{-9*}	0.81	0.76–0.87
rs521660 G>A	3rd	762	55.3	515	37.3	102	7.4	1379	627	49.1	530	41.504	120	9.4	1277	10.9	9.4×10 ⁻⁴	0.82	0.72–0.92
	Replication	1197	55.9	781	36.5	162	7.6	2140	1597	49.3	1342	41.445	299	9.2	3238	21.8	3.1×10 ⁻⁶	0.81	0.75–0.89
	Combined															32.7	1.2×10 ^{-8*}	0.81	0.76–0.88

Abbreviations: CI, confidence interval; MI, myocardial infarction; OR, odds ratio; SNP, single nucleotide polymorphism.
^{*}P-values were calculated by Mantel-Haenszel test.

Table 4 Haplotype analysis

Haplotype	SNP IDs Risk allele			Haplotype frequency		Comparison of haplotype frequency	
	rs490556	rs11748327	rs521660	Case	Control	χ ²	P-value
	T	C	G				
Haplotype 1	T	C	G	0.727	0.688	30.7	3.0×10 ⁻⁸
Haplotype 2	C	T	A	0.176	0.213	37.1	1.1×10 ⁻⁹
Haplotype 3	C	C	A	0.053	0.058	1.4	2.3×10 ⁻¹
Haplotype 4	T	T	A	0.031	0.030	0.3	6.0×10 ⁻¹

Abbreviation: SNP, single nucleotide polymorphism.

miRNA-mediated translational suppression.²⁵ Therefore, the SNPs on chromosome 5p15.3 might be located within the region encoding unidentified miRNAs, affect their functions, and contribute to the development and/or progression of CAD. Although it is very difficult to reveal function of the SNP with the present knowledge, we think the increasing attention to function and/or higher-order structure of genome and subsequent progress will help to solve this problem.

We believe that knowledge of genetic factors contributing to its pathogenesis provides a useful clue for the development of diagnostic methods, treatments and preventive measures for this common but serious disorder.

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

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Rationale: Grb2-associated binder (Gab) docking proteins, consisting of Gab1, Gab2, and Gab3, have crucial roles in growth factor-dependent signaling. Various proangiogenic growth factors regulate angiogenesis and endothelial function. However, the roles of Gab proteins in angiogenesis remain elusive.

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* (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.

Conclusion: Gab1 is essential for postnatal angiogenesis after ischemia via HGF/c-Met signaling. (*Circ Res.* 2011; 108:664-675.)

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 Src homology-2 (SH2) domain-containing

proteins such as SH2 containing protein tyrosine phosphatase 2 (SHP2), phosphatidylinositol 3-kinase regulatory subunit p85, phospholipase C γ , 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

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display embryonic lethality with impaired development of heart, placenta, skin, and skeletal muscle.^{3,4} Conventional Gab2 knockout (Gab2KO) mice do not show any obvious developmental defects, but display impaired allergic responses, osteoclast defects, and abnormal hematopoiesis in adulthood.⁵⁻⁷ Gab3 knockout mice exhibit no obvious phenotype.⁸ Because Gab1KO mice are embryonic lethal, we and others created conditional knockout mice of Gab1 using the *Cre-loxP* system.⁹⁻¹² 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.¹¹

Angiogenesis, the process of new blood vessel formation, is involved in many physiological and pathological settings such as ischemia, atherosclerosis, diabetes, and cancer.¹³ 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 processes at the EC-EC interfaces establish a continuous lumen and blood flow promote maturation processes such as the "stabilization" of cell junctions and tight pericyte recruitment.^{14,15} The angiogenic growth consists of these multistep processes from "endothelial sprouting" to "endothelial stabilization." ERK5 has been reported to have a central role for flow-mediated stabilization via upregulation of endothelial stabilization factor Krüppel-like factor (KLF)2.^{16,17} However, the molecular mechanism how ERK5-KLF2 pathway is activated in in vivo angiogenesis remains unclear to date.

We reported an important role of Gab1 for ERK5 activation in gp130-dependent cardiomyocyte hypertrophy.^{18,19} On the other hand, it has been reported that Gab1 has a role for VEGF-dependent signaling in the in vitro experiments using ECs.²⁰⁻²² However, the in vivo role of Gab proteins in angiogenesis has not been addressed to date. Here, we demonstrate that Gab1 in the vascular endothelium is essential for postnatal angiogenesis after ischemia. Endothelium-specific deletion of Gab1 results in enhanced propensity to limb necrosis after hindlimb ischemia (HLI) caused by impaired angiogenesis via hepatocyte growth factor (HGF)/c-Met signaling. On the contrary, global deletion of Gab2, another Gab protein expressed in the vascular endothelium, does not lead to limb necrosis and impairment of blood flow recovery after HLI compared with control mice. Consistently, Gab1, but not Gab2, is required for activation of ERK1/2, ERK5, and AKT after stimulation with HGF in ECs. Gab1 associates with SHP2 and p85 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>.

Non-standard Abbreviations and Acronyms

β-gal	β -galactosidase
CA	constitutively active
DN	dominant-negative
EC	endothelial cell
Egr	early growth response
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
Gab	Grb2-associated binder
Gab1ECKO	endothelium-specific Gab1 knockout
Gab1KO	conventional Gab1 knockout
Gab2KO	conventional Gab2 knockout
GST	glutathione S-transferase
HGF	hepatocyte growth factor
HLI	hindlimb ischemia
HUVEC	human umbilical vein endothelial cell
KLF	Krüppel-like factor
LDBF	laser Doppler blood flow
MACS	Magnetic Cell Sorting
MAPK	mitogen activated protein kinase
MEF2C	myocyte enhancer factor 2
MEK	mitogen activated protein kinase/extracellular signal-regulated kinase
SHP2	Src homology-2-containing protein tyrosine phosphatase 2
siRNA	small interfering RNA
TM	thrombomodulin
VEGF	vascular endothelial growth factor

Animals

Gab1^{lox} mice were generated in 129/Sv-C57BL/6J mixed background as described previously.¹¹ *Tie2-Cre* transgenic mice in CD-1 background were provided from Dr Thomas N. Sato.²³ Endothelium-specific Gab1 knockout (Gab1ECKO) mice were generated by crossing *Gab1^{lox/lox}* mice with *Tie2-Cre* transgenic mice. The creation of Gab2KO (*Gab1^{lox/lox} Gab2^{-/-}*) mice were also described previously.¹¹ All the animals used for the experiments were 7- to 8-week-old male mice maintained on a 129/Sv-C57BL/6J-CD-1 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 rDNA 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 Gab1ECKO mice using the *Cre-loxP* system. We created a *Gab1^{lox}* allele by introducing 2 *loxP* sites into introns flanking exon 2 which encodes part of the pleckstrin homology domain as described previously.¹¹ To cause recombination of the floxed allele exclusively in EC lineage, mice homozygous for the *Gab1-loxP*-targeted allele (*Gab1^{lox/lox}*) were crossed with transgenic mice expressing *Tie2* promoter-driven *Cre* recombinase (*Tie2-Cre* mice).²³ We created Gab1ECKO (*Gab1^{lox/lox} Tie2-Cre(+)*) mice by crossing

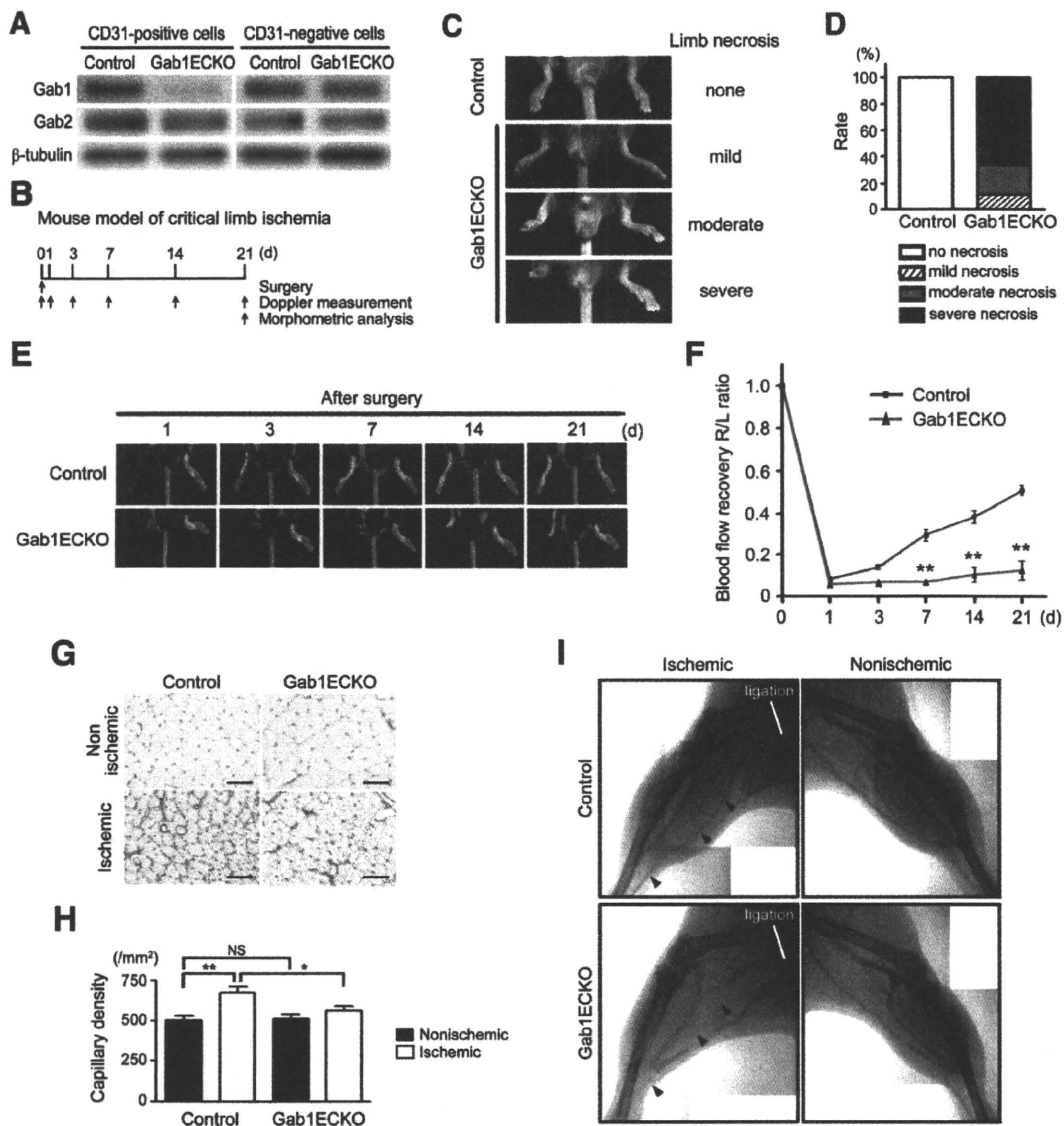


Figure 1. Impaired blood flow recovery and angiogenesis in Gab1ECKO mice. **A**, Gab1 was successfully ablated in the ECs in Gab1ECKO mice. The CD31-positive ECs were purified from the limb muscles using the MACS system. Whereas the expression of both Gab1 and Gab2 in the CD31-negative cells was almost 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 day1, 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 \pm SEM. ** P <0.01 vs control. **G**, Representative CD31 staining of capillaries from sections of nonischemic and ischemic adductor muscles. Scale bar, 100 μ m. **H**, Quantitative analysis of capillary density in control and Gab1ECKO mice (number per high-power field; \times 400 magnification). Values are shown as means \pm 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^{+/*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%); *Gab*^{+/*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

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^{fllox/fllox}*) mice with *Gab2^{-/-}* mice to create *Gab1^{fllox/fllox} 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-

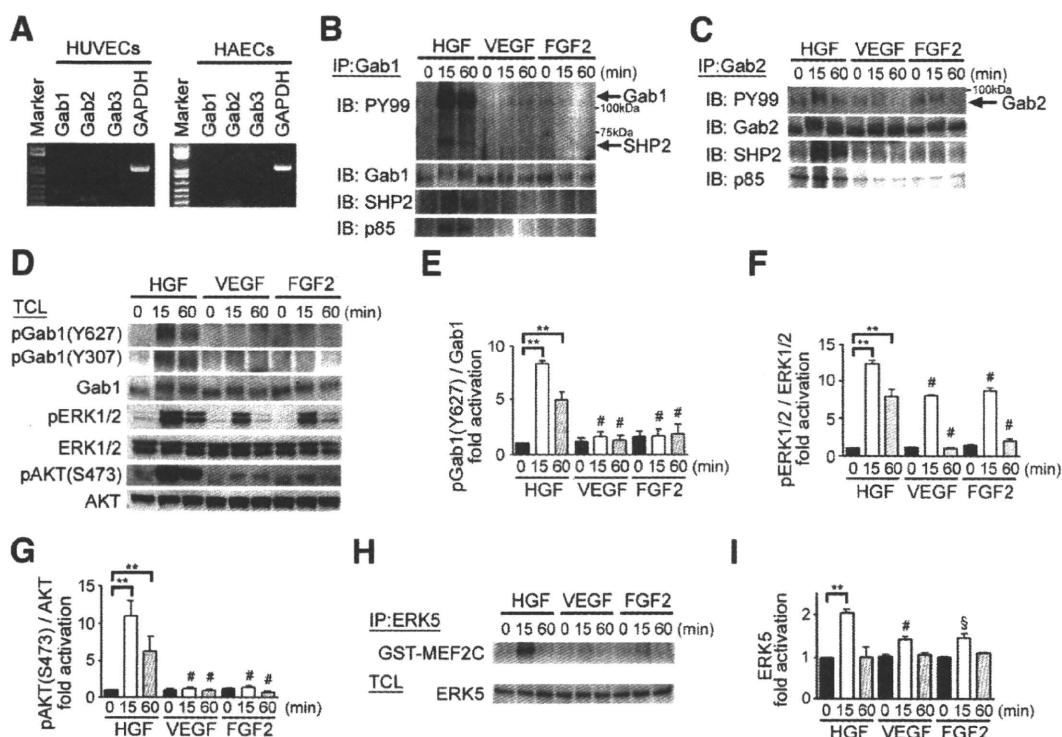


Figure 2. Gab1 and its downstream signaling pathways are strongly activated after stimulation with HGF in HUVECs. **A**, RT-PCR shows the expression of Gab1 and Gab2 mRNAs, but not Gab3 mRNA, in both HUVECs and human aortic ECs (HAECs). **B** and **C**, Tyrosine phosphorylation of Gab1 (**B**) and Gab2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

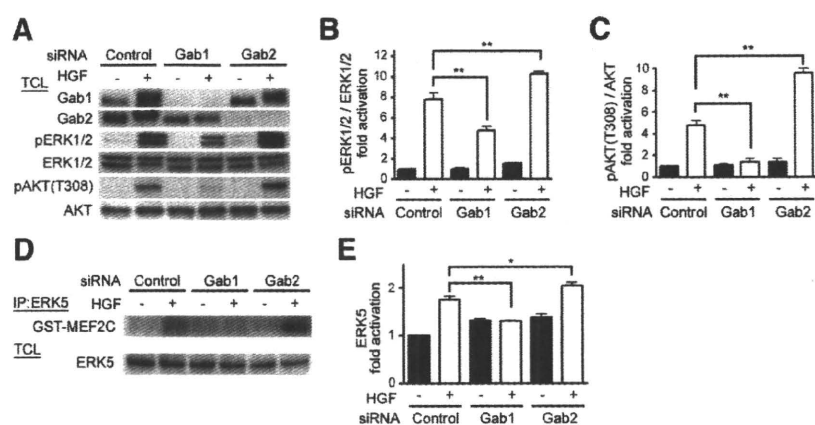


Figure 3. siRNA-mediated knockdown of Gab1, but not Gab2, significantly attenuates activation of ERK1/2, AKT, and ERK5 in response to HGF in HUVECs. **A**, HUVECs were transfected with control siRNA (Control) or siRNAs targeting either Gab1 or Gab2 for 48 hours. After serum starvation, HUVECs were treated 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). 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<0.05, **P<0.01 for the indicated groups. Values are shown as means±SEM for 3 separate experiments.

Gab1^{ΔSHP2} compared with control cells, but not in cells expressing Gab1^{Δp85} (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^{ΔSHP2}, or Gab1^{Δp85}, 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^{ΔSHP2}, compared with control cells expressing β-gal (Figure 4F and 4G). In addition, overexpression of Gab1^{Δp85} 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^{ΔSHP2}. Overexpression of MEK1^{CA}, but not MEK5^{CA}, restored cell migration of the HUVECs overexpressing of Gab1^{ΔSHP2} (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^{ΔSHP2} or Gab1^{Δp85} (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^{ΔSHP2} or Gab1^{Δp85}, 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^{ΔSHP2} (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

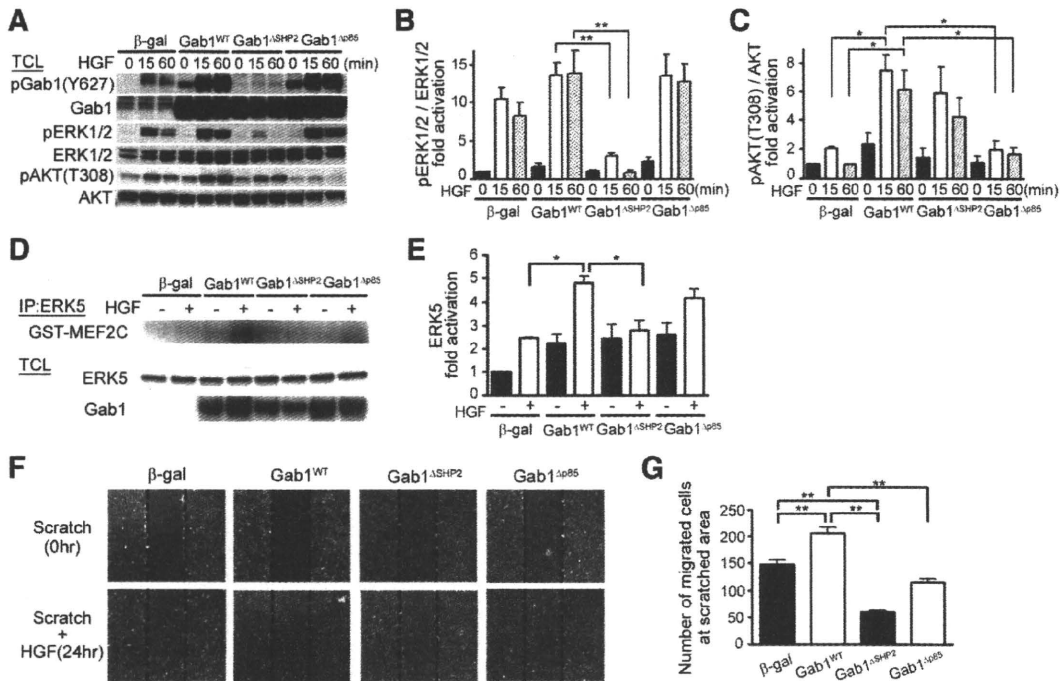


Figure 4. Gab1 is essential for activation of ERK1/2, AKT, and ERK5 and subsequent cell migration after HGF stimulation in HUVECs. **A**, HUVECs, infected with the indicated adenovirus vectors, were stimulated with HGF (20 ng/mL). Cell lysates were collected and subjected to immunoblotting analyses using the antibodies indicated at the left. Expression level of Gab1 was almost comparable in the cells overexpressing Gab1^{WT}, Gab1^{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^{flx/flx}), 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

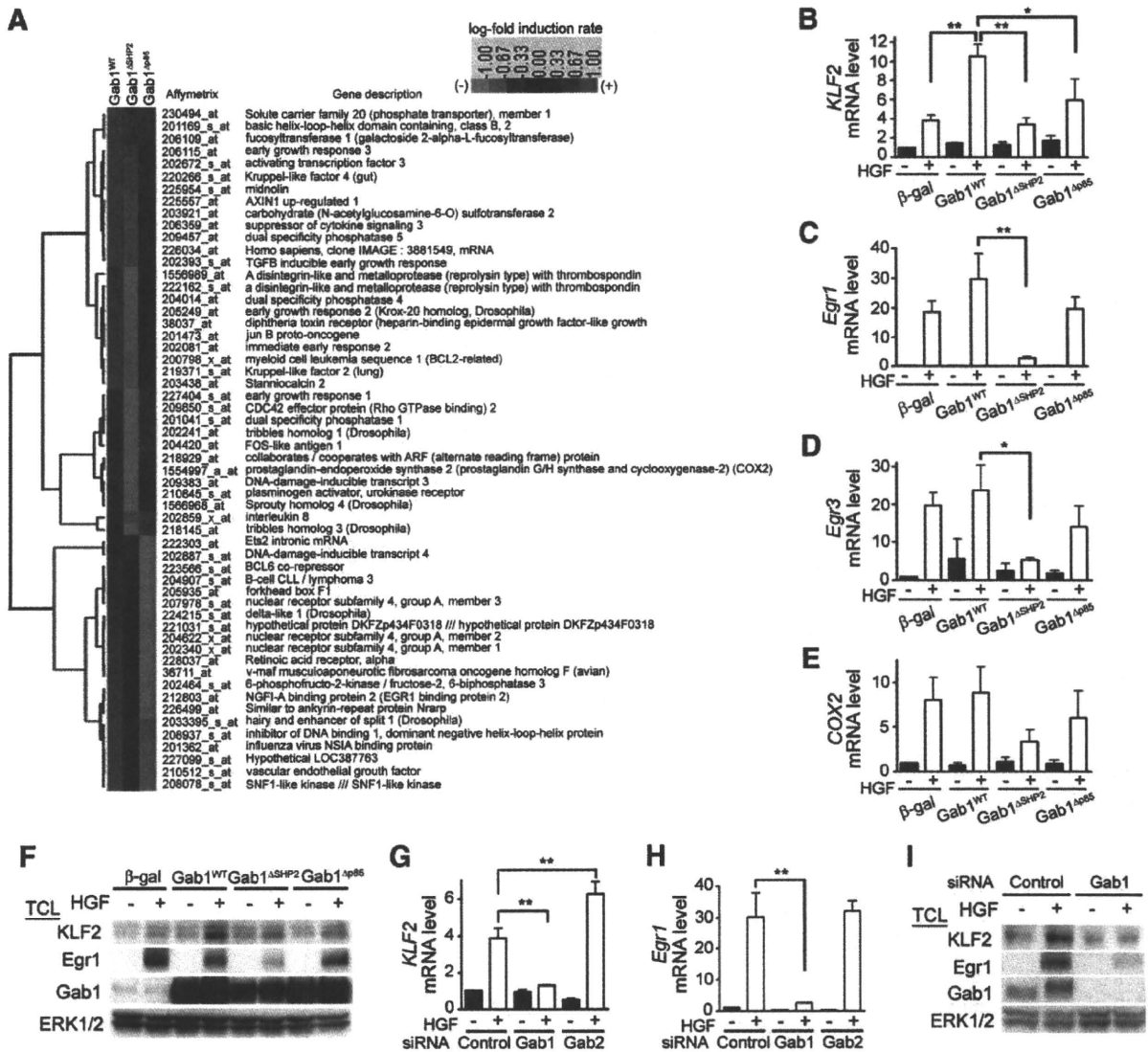


Figure 5. HGF stimulation leads to a distinct pattern of gene expression via Gab1. **A**, HUVECs infected with the indicated adenovirus vectors were serum-starved and treated with vehicle or 20 ng/mL HGF for 1 hour. Total RNA was purified from the HUVECs and subjected to Affymetrix microarray analysis.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

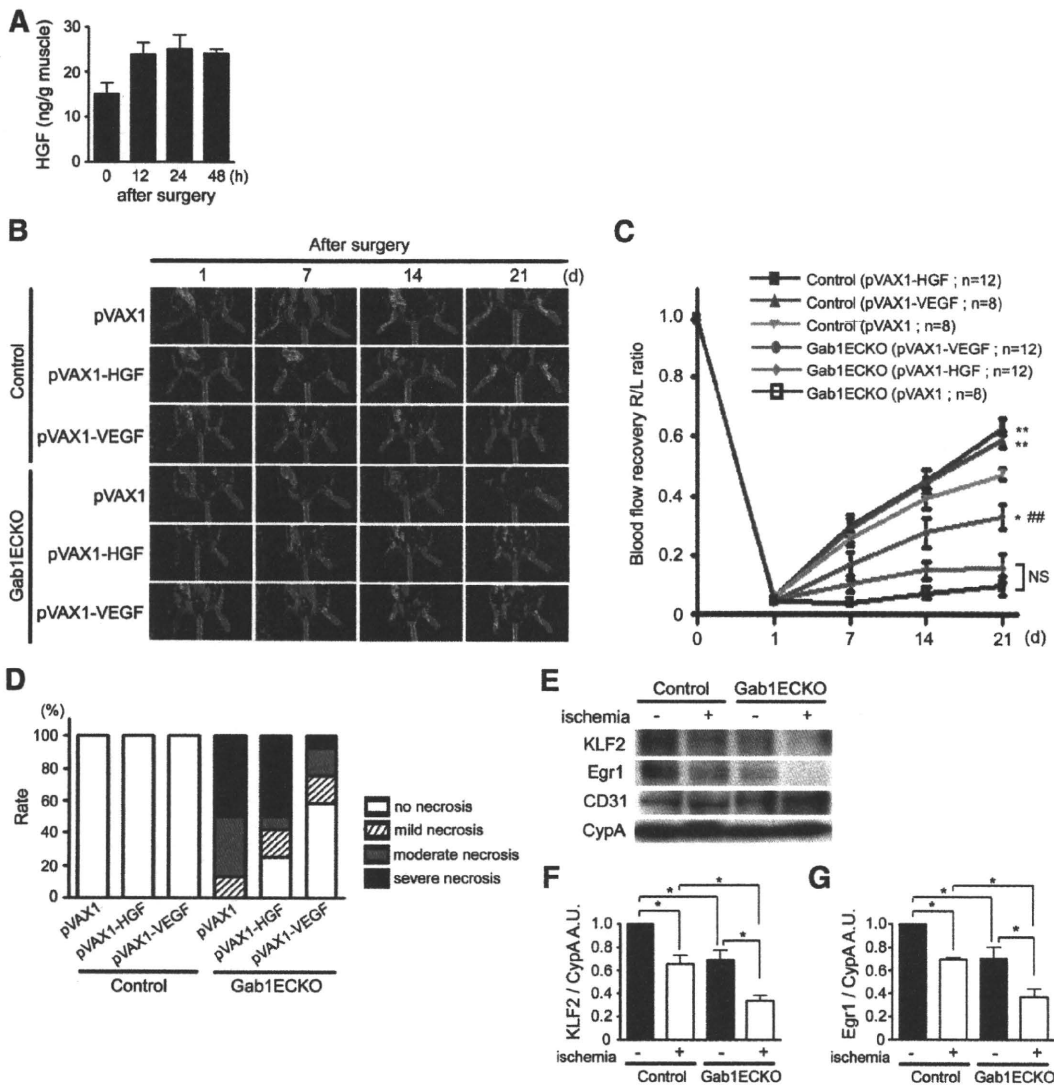


Figure 6. Gene transfer of VEGF, but not HGF, partially ameliorates the limb necrosis after HLI in Gab1ECKO mice. **A**, Concentration of HGF in the ischemic limb muscle was examined by ELISA (n=3). **B**, Representative LDBF images of mice, injected with control, HGF, and VEGF expression plasmids after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

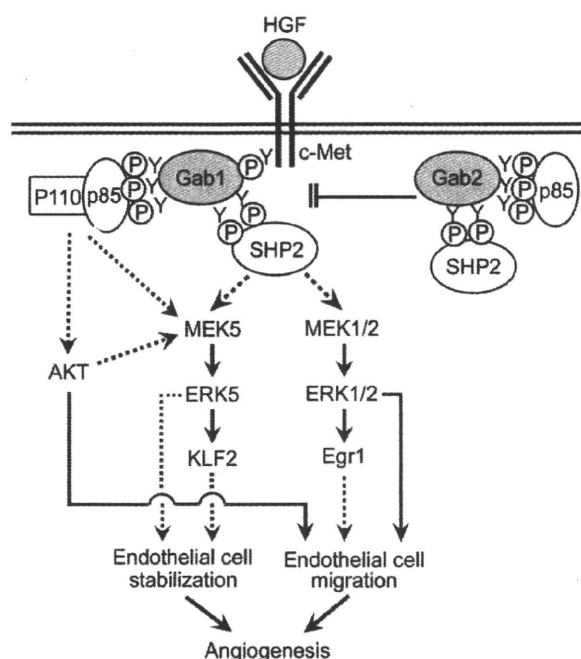


Figure 7. Schematic illustrations of the role of Gab1 in postnatal angiogenesis. HGF induces tyrosine phosphorylation of both Gab1 and Gab2 in the ECs. Both Gab1 and Gab2 associate with SHP2 and p85 on stimulation with HGF. Gab1 is required for activation of ERK1/2, ERK5, and AKT in response to HGF.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