FHL-2 Suppresses VEGF-Induced Phosphatidylinositol 3-Kinase/Akt Activation via Interaction With Sphingosine Kinase-1

Hiroki Hayashi, Hironori Nakagami, Yoichi Takami, Hiroshi Koriyama, Masaki Mori, Katsuto Tamai, Jianxin Sun, Kaori Nagao, Ryuichi Morishita, Yasufumi Kaneda

Objective—In the functional screening of a human heart cDNA library to identify a novel antiangiogenic factor, the prime candidate gene was “four-and-a-half LIM only protein-2” (FHL-2). The goal of this study is to clear the mechanism of antiangiogenic signaling of FHL-2 in endothelial cells (ECs).

Methods and Results—Overexpressed FHL-2 strongly inhibited vascular endothelial growth factor (VEGF)-induced EC migration. In the angiogenic signaling, we focused on sphingosine kinase-1 (SK1), which produces sphingosine-1-phosphate (S1P), a bioactive sphingolipid, as a potent angiogenic mediator in ECs. Immunoprecipitation and immunostaining analysis showed that FHL-2 might bind to SK1. Importantly, overexpression of FHL-2 in ECs inhibited VEGF-induced SK1 activity, phosphatidylinositol 3-kinase activity, and phosphorylation of Akt and eNOS. In contrast, overexpression of FHL-2 had no effect on S1P-induced Akt phosphorylation. Interestingly, VEGF stimulation decreased the binding of FHL-2 and SK1. Depletion of FHL-2 by siRNA increased EC migration accompanied with SK1 and Akt activation, and increased the expression of VEGF receptor-2 which further enhanced VEGF signaling. Furthermore, injection of FHL-2 mRNA into Xenopus embryos resulted in inhibition of vascular network development, assessed by in situ hybridization with endothelial markers.

Conclusions—FHL-2 may regulate phosphatidylinositol 3-kinase/Akt via direct suppression of the SK1-S1P pathway in ECs. (Arterioscler Thromb Vasc Biol. 2009;29:909-914.)

Key Words: FHL-2 ■ VEGF ■ sphingosine kinase-1 ■ endothelial cells ■ Akt pathway

Angiogenesis is critical for organ growth and development, wound healing, and reproduction.1 Therapeutic modulation of angiogenesis using angiogenic growth factors, such as vascular endothelial growth factor (VEGF), has been proposed for revascularization in ischemic diseases and has investigated in clinical trials.2 Angiogenesis is ultimately controlled by a balance between endogenous pro- and antiangiogenic molecules, which may include various angiogenic peptides, hormone metabolites, and apoptosis modulators.13

We recently developed a functional gene screening system with the HVJ-E vector4 and screened a human heart cDNA library for antiangiogenic genes. One of the candidates was “four and a half LIM domain protein-2” (FHL-2/SLIM3), a member of the LIM-only subclass of the LIM protein superfamily. LIM proteins are defined by the presence of one or more LIM proteins that mediate protein-protein interactions.5 The members of the FHL subclass of LIM-only proteins consist of four and a half LIM domains, are expressed primarily in cardiac tissues, and mainly function as transcriptional cofactors.6–10 Heart development and function is normal in FHL-2-deficient mice,11 but FHL-2 deficient mice develop cardiac hypertrophy in response to β-adrenergic stimulation.12

Although FHL-2 is present in a human heart cDNA library, we identified the endogenous expression in vascular endothelial cells (ECs). The goal of the present study was to clarify the role of the FHL-2 gene in angiogenic signaling in ECs.

Materials and Methods

Northern Blotting, Western Blotting, and Immunohistochemical Staining

Northern blotting, Western blotting and immunohistochemical staining were performed as previously described.13 In immunoprecipitation

Received October 7, 2008; revision accepted March 8, 2009.

From the Division of Gene Therapy Science (H.H., H.N., Y.T., H.K., M.M., K.T., Y.K.), the Department of Geriatric Medicine (Y.T., H.K.), and the Division of Clinical Gene Therapy and Graduate School of Medicine (K.N., R.M.), Osaka University, Japan; and the Department of Cell Biology and Molecular Medicine (J.S.), UMDNJ-New Jersey Medical School, Newark.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.108.178541

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assay, the interaction between FHL-2 and SK1 was evaluated by immunoblotting with anti-M2 flag antibody (Sigma), anti-HA antibody (Sigma), anti-SK1 antibody (Abgent), and anti-FHL-2 (MBL). Primary antibody used for the immunochemical staining was anti-FHL-2 (Abcam) antibody at 4°C overnight. Corresponding secondary antibodies were labeled with peroxidase (GE healthcare).

In Vivo Xenopus Embryo mRNA Injection Model

Xenopus laevis embryos were generated using standard techniques and staged as previously described. Whole mount in situ hybridization was performed as described previously described, using the DIG RNA Labeling kit (Roche, Basel, Switzerland). To evaluate vascular formation of Xenopus embryos, embryos were hybridized with probes for endothelial marker, Xenopus fli-1, or msr.

Statistical Analysis

All values are expressed as mean±SD. Analysis of variance with subsequent Fisher PLSD test or the unpaired Student t test was used to determine the significance of differences in multiple comparisons. All statistical analysis was performed using Stat-View 5.0 software (SAS Institute Inc). Values of P<0.05 were considered to represent statistical significance.

Results

Expression of FHL-2 in Vascular ECs

We screened a human heart cDNA library using the HVJ-E functional screening system as previously reported to identify the novel antiangiogenic modulators. One candidate of antiangiogenic modulator genes was FHL-2, which is initially identified to be highly expressed in cardiac tissue. Northern blot analysis using human cardiovascular tissue showed that FHL-2 mRNA was detected in the aorta as well as in heart (supplemental Figure IA). Similarly, immunohistochemical staining demonstrated that FHL-2 was expressed in coronary arteries and cardiac myocytes. In the ascending aorta, the expression of FHL-2 was detected both in ECs and smooth muscle cells (SMCs; supplemental Figure IB). Western blot showed that FHL-2 was fairly expressed in human aorta ECs (HAECs) and HASMCs, less in THP-1 (supplemental Figure IC). Several growth factors (ie, vascular endothelial growth factor [VEGF] 25 ng/mL, fibroblast growth factor-2 [FGF2] 25 ng/mL, epidermal growth factor [EGF] 25 ng/mL, VEGF 25 ng/mL, +FGF2 25 ng/mL, +EGF 25 ng/mL) did not change the expression of FHL-2. However, expression of FHL-2 was slightly decreased by stimulation with tumor necrosis factor-α (TNF-α) 10 ng/mL (supplemental Figure ID).

FHL-2 Attenuates EC Growth and Interacts With SK1 in ECs

FHL-2 was previously identified as an antiproliferative factor. We confirmed that FHL-2 suppressed VEGF-induced cell growth in BAECs by using MTS assay and c-fos promoter assay (supplemental Figure IIA and IIB). FHL-2 also repressed VEGF-induced cell growth in HAECS (supplemental Figure IIC). FHL-2 interacting molecules may elucidate the mechanism of antiangiogenic effect of FHL-2. Importantly, previous studies reported that sphingosine kinase-1 (SK1) bound to FHL-2 and produce sphingosine-1-phosphate (S1P), a bioactive sphingolipid that acts as a potent angiogenic mediator in ECs.

Consistent with the previous report in cardiac myocytes, immunostaining analysis in BAECs showed that overexpressed HA-FHL-2 and Flag-SK1 were colocalized (Figure IA). Immunoprecipitation experiments demonstrated binding of FHL-2 to SK1 in BAECs with overexpressed HA-FHL-2 and Flag-SK1. Interestingly, treatment of BAECs with VEGF 50 ng/mL decreased direct interaction between FHL-2 to SK1 at 5 minutes after stimulation (supplemental Figure IIIA). Moreover, immunoprecipitation showed that endogenous interaction of FHL-2 and SK1 was decreased by stimulation with VEGF 50 ng/mL in HAECs (Figure 1B). However, the stimulation of VEGF 50 ng/mL did not change the expression level of these mRNA and proteins in HAECs (supplemental Figure IIIB and IIIC). These results indicated that FHL-2 interacted with SK1 in ECs.

FHL-2 Suppresses SK1 Activity, Which Regulates VEGF-Induced Akt Phosphorylation and EC Migration

Overexpression of FHL-2 in BAECs strongly suppressed VEGF-induced SK1 activation and S1P production at 5 or 10 minutes after stimulation (Figure 2A). Treatment of HAECS with VEGF or S1P activated Akt phosphorylation, whereas pretreatment with SK1 inhibitor, DMS (dimethylsphingosine), or S1P receptors antagonist, VPC23019, suppressed Akt phosphorylation induced by VEGF (supplemental Figure IA and IVB). It is known that SK1-S1P signaling strongly contributes to EC migration, which is
essential for angiogenic processes. DMS or VPC23019 strongly suppressed VEGF-induced migration in HAECs accompanied with Akt phosphorylation (supplemental Figure IVC and IVD). Inversely, S1P-induced cell migration was not inhibited by treatment with DMS (supplemental Figure IVC).

Similarly, overexpression FHL-2 in BAECs inhibited VEGF (50 ng/mL)-induced Akt and eNOS phosphorylation (Figure 2B). Further analysis showed that VEGF-induced Akt phosphorylation was significantly inhibited by overexpressed FHL-2 from 10 minutes to 20 minutes in BAECs (10 minutes: 72% inhibition, 15 minutes: 71% inhibition, 20 minutes: 57% inhibition, compared to control, respectively, supplemental Figure IVE). Overexpressed FHL-2 also attenuated VEGF-induced phosphatidylinositol 3-kinase activity associated with p85 and p110 (supplemental Figure VA). However, S1P-induced Akt phosphorylation was not suppressed by overexpression of FHL-2 in BAECs (Figure 2C). Similarly, VEGF or S1P induced the S1P receptors activity assessed by [35S] GTPγS binding assay, which measures GDP-GTP exchange on the Gαi subunit by coupling of S1P receptors and G proteins. Overexpressed FHL-2 suppressed VEGF-induced S1P receptors activation but not S1P-induced activation (supplemental Figure VB). Moreover, overexpressed FHL-2 strongly inhibited VEGF-induced migration, but not S1P-induced migration in BAECs (Figure 2D) and HAECS (supplemental Figure VC). Scratch wound assay also demonstrated that overexpressed FHL-2 markedly inhibited EC migration (Supplement Figure VD). These results suggested that FHL-2 strongly suppressed VEGF-induced EC migration, accompanied with the inhibition of Akt and eNOS phosphorylation.

Depletion of FHL-2 Enhances SK1 Activity and EC Growth
To investigate endogenous function of FHL-2 in ECs, we designed 3 different siRNAs for FHL-2 knockdown in ECs. Transfection of FHL-2 439-siRNA strongly reduced FHL-2 expression and 511-siRNA mildly decreased it in ECs (supplemental Figure VIA). Knockdown of FHL-2 expression by transfection of 439-siRNA increased SK1 activity, to a similar level to VEGF treatment, however transfection of 511-siRNA did not (Figure 3A). Transfection of 439-siRNA, but not 511-siRNA, induced Akt phosphorylation (Figure 3B). Similarly, transfection of 439-siRNA, but not 511-siRNA, increased EC migration compared to control or scramble siRNA (Figure 3C), and also increased EC growth compared with control siRNA (119% increase, data not shown). These results indicated that endogenous FHL-2 might have antiangiogenic effect in ECs by regulation of SK1 activity.

Depletion of FHL-2 Enhances the Expression of VEGFR2
We also analyzed the effect of FHL-2 on VEGF receptor activation in VEGF signaling pathway. Overexpressed FHL-2 or pretreatment of SK1 inhibitor, DMS, did not affect the phosphorylation of VEGFR2 (VEGFR receptor-2) induced by VEGF or S1P (Figure 4A and supplemental Figure VIB and VIC). However, depletion of FHL-2 enhanced the expression of VEGFR2, accompanied with upregulation of its phosphorylation (Figure 4B). Similarly, depletion of FHL-2 enhanced...
VEGF-induced phosphorylation of Akt as well as ERK (supplemental Figure VID). We further examined whether depletion of FHL-2 could increase VEGFR2 expression via SK1-S1P pathway. As shown in Figure 4C, depletion of FHL-2 increased the expression of VEGFR2, however co-treatment of the S1P receptors antagonist, VPC23019, did not attenuate it (Figure 4C), which suggests that the regulation of VEGFR2 expression by FHL-2 might be independent of S1P signaling. Importantly, depletion of FHL-2 also increased Akt phosphorylation, phosphatidylinositol 3-kinase activity associated with p85 and p110, S1P receptors activity, which were abolished by cotreatment of S1P receptors antagonist, VPC23019 (Figure 4C and supplemental Figure VIE and VIF). Thus, the activation of phosphatidylinositol 3-kinase/Akt pathway by depletion of FHL-2 may be dependent on SK1-S1P pathway.

FHL-2 Inhibits Vascular Formation in Xenopus Embryos

The SK1-S1P pathway has been implicated in vasculogenesis and vascular maturation.20–23 FHL-2 mRNA was injected into Xenopus embryos to examine the effect in the development of vascular formation. Expression of FHL-2 and SK1 in Xenopus embryo was verified using RT-PCR analysis (supplemental Figure VII).

To evaluate vascular formation in Xenopus embryos, we examined the expression patterns of endothelial markers, fli-1 and msr. Fli is a member of the ETS-family and shown to be expressed in cranial neural crest cells, angioblasts and endothelial cells of the forming blood vessels.24 Msr (Mesenchyme-associated serpentine receptor) is a member of the G protein-coupled receptor in Xenopus and homologue to the human APJ receptor.25 This gene also traces an endothelial lineage and represents a very early and unique marker in Xenopus of the specification of vascular endothelia.26 Whole mount in situ hybridization showed a reduced staining for these endothelial markers in stage 32 to 33 embryo injected with FHL-2 mRNA. We quantified this antiangiogenic effect in Xenopus based on the previous report.27 As shown in the Table, we counted the numbers of embryos showing a reduction in the vitelline vein network (Figure 5, circle) or intersomitic veins (Figure 5, arrowhead), and demonstrated the increased embryos with angiogenic defects by injection of FHL-2 mRNA. These results suggest that injection of FHL-2 resulted in the inhibition of vasculogenesis.

Discussion

The present study demonstrated that FHL-2 inhibited SK1 activity in vascular ECs and that the interaction of FHL-2 with SK1, which was regulated by VEGF, played an important role in the VEGF-induced signaling.

S1P, a bioactive lipid mediator produced by activated-SK1, exerts a variety of actions in many types of cells, including vascular ECs.28 For example, S1P stimulates proliferation,28

Table. The Effect of FHL-2 Overexpression on Vascular Formation in Xenopus Embryos

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AD indicates angiogenic defect. *No. of embryos with AD/No. of embryos analyzed ×100. Xfli-1 indicates Xenopus fli-1. Xmsr indicates Xenopus mesenchyme-associated serpentine receptor; None, no treatment with embryos; GFP, injection of GFP mRNA into embryos; FHL-2, injection of FHL-2 mRNA into embryos. Embryos were analyzed by in situ hybridization with probes for Xenopus endothelial markers.
FHL-2 is not fully understood, it has been reported that FHL-2 identified the expression and function of FHL-2 in ECs. Phosphatidylinositol 3-kinase/Akt activity via SK1-S1P pathway, although the mechanism is still unknown in detail, our results suggest the complexity of this regulatory mechanism. Additionally, FHL-2 did not affect the VEGFR2 expression, which might indicate that FHL-2 is a multifunctional protein with numerous binding targets including receptors and transcriptional factors, which may possibly regulate the transcription of VEGFR2. Furthermore, overexpressed FHL-2 did not affect the VEGFR2 expression, which might suggest the complexity of this regulatory mechanism. Although the mechanism is still unknown in detail, our results demonstrated that FHL-2 might regulate VEGF-induced phosphatidylinositol 3-kinase/Akt activity via SK1-S1P pathway and VEGFR2 expression. In our present study, we identified the expression and function of FHL-2 in ECs. However, it seemed that FHL-2 is expressed not only in ECs but also in SMCs in aorta. Although the function of FHL-2 in SMCs is not fully understood, it has been reported that FHL-2 might contribute the phenotypic switching of SMCs. FHL-2 was upregulated in an SRF-dependent manner during ES cell differentiation and in response to RhoA activation. Importantly, FHL-2 was shown to inhibit serum response factor (SRF)-dependent transcription of SMC-specific genes by competing with myocardin-related transcriptional factors (MRTFs)-A for SRF binding in 293T cells. SRF as well as MRTFs are recognized as liaisons connecting the key pathways of arteriogenesis, ie, Rho pathway with downstream genes. Therefore, we speculate that FHL-2 in vascular SMCs might contribute the process of atherosclerosis or angiogenesis through suppressing phenotypic switching.

In conclusion, FHL-2 is expressed in vascular ECs, where it may exert VEGF-induced phosphatidylinositol 3-kinase/Akt through inhibition of SK1.

Sources of Funding
This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H.H. and H.N.) and by the Japan Heart Foundation Grant for Research (H.N.).

Disclosures
None.

References

25. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, Shi X,


18. Limaye V, Li X, Hahn C, Xia P, Berndt MC, Vadas MA, Gamble JR.

28. Saba JD, Hla T. Point-counterpoint of sphingosine 1-phosphate metabo-


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June 2009


17. Sun J, Yan G, Ren A, You B, Liao JK. HFL2/SLIM3 decreases cardio-

276:31780–31785.

339–345.

2006;99:933–9841.


1800–1803.

19672–19677.

545–558.

315x712]. 2001;276:12420–12426.

301–312.


339–345.


1995;


229:3994–3999.

147:455–545.


47. Deindl E. Arteriogenesis: a focus on signal transduction cascades and

32. Igarashi J, Bernier SG, Michel T. Sphingosine 1-phosphate and activation

381x693]. 2001;276:12420–12426.


1933;59:129–140.


47. Deindl E. Arteriogenesis: a focus on signal transduction cascades and

2006;6:63.

2006;276:31780–31785.


43. Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S, Nakamura S.

36. Fukuda Y, Kihara A, Igarashi Y. Distribution of sphingosine kinase


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Arterioscler Thromb Vasc Biol. 2009;29:909-914; originally published online March 26, 2009;
doi: 10.1161/ATVBAHA.108.178541

Arteriosclerosis, Thromosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/6/909

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In the article that appeared on page 909 of the 29.6 issue, a second corresponding author was not included in the footnote. The second corresponding author’s name is Yasufumi Kaneda and the email for correspondence is kaneday@gts.med.osaka-u.c.jp. The error has been noted in the online version of the article, which is available at http://atvb.ahajournals.org/.

Reference:
Supplementary Figure I

A

B

C

D
Supplementary figure II

A

![Cell growth (fold increase over basal)]

B

![C-fos promoter activity (fold increase over basal)]

C

![Cell growth (fold increase over basal)]
Supplementary figure III

A

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B

Relative expression (fold increase over basal)

C

Relative expression (fold increase over basal)
Supplementary Figure IV

A. VEGF and VEGF+DMS (min) phospho-Akt Akt

B. VEGF and VEGF+VPC23019 (min) phospho-Akt Akt

C. p<0.0001 N.S.

D. no treatment VEGF VEGF+VPC23019

E. control FHL-2 (min) phospho-Akt Akt

p<0.0001
Supplementary figure V

A  

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p<0.0001

N.S.

number of cells

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Supplementary Figure VI

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β-actin

B

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IB: VEGFR2

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phospho-Akt
Akt
phospho-ERK
ERK
β-actin

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GTPγS binding to Gαi (fold increase over basal)

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Supplementary figure VII

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Supplement Method

Chemical reagent, cell culture and transfection

Human recombinant vascular endothelial growth factor (VEGF) fibroblast growth factor-2 (FGF2), epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α) were obtained from Pepro Tech EC LTD (London, UK). Sphingosine-1-phosphate and SK1 inhibitor, DMS (Dimethylsphingosine), were obtained from BIOMOL (Plymouth Meeting, PA). S1P receptors antagonist, VPC23019, was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). HAEC (human aortic endothelial cells) were purchased from Clonetics Corp. (Palo Alto, CA) and were maintained as previously described.1 BAEC (bovine aortic endothelial cells) were purchased from Clonetics Corp and the American Type Culture Collection (ATCC), respectively, and were maintained as previously described.1 Transfection of plasmid DNA was performed using LipofectAMINE2000 (Invitrogen, Grand Island, NY) or Amaxa (amaxa biosystems, USA) according to manufacturer’s instruction.

FHL-2 knockdown

The siRNA for FHL-2 was designed by SiSniper (Mitsubishi Space Software, Amagasaki, Japan) and constructed by Nippon EGT (Toyama, Japna). FHL-2 siRNA or scramble siRNA was transfected into BAEC using LipofectAMINE 2000.

FHL-2 254-siRNA sense: UCGGAACCAAGAGCUUCAUTT
FHL-2 254-siRNA antisense: AUGAAGCUCUUGGUUCCGATT
FHL-2 439-siRNA sense: CCCGCAAGAUGGAGUACAATT
FHL-2 439-siRNA antisense: UUGUACUCCAUGUUGCGGGTT
FHL-2 511-siRNA sense: UCGGAACCAAGAGCUUCAUTT
FHL-2 511-siRNA antisense: AUGAAGCUCUUGGUUCCGATT
scramble siRNA sense: GAACUGUUCCAGUCAGCUUTT
scramble siRNA antisense: AAGCUGACUGGAACAGUUCTT

**Northern blotting**

Equal aliquots of total RNA (15 µg) obtained from HAEC (RNeasy, Qiagen, Valencia, CA) were separated by 1% formaldehyde-agarose gel electrophoresis, transferred to membranes (Amersham Biosciences, Freiburg, Germany), and hybridization and washing were performed as previously described. Northern analysis was also performed with human cardiovascular blot membrane (Human Cardiovascular System MTN® Blot) prepared from high-quality poly(A)+ RNA normalized for a β-actin hybridization signal from Clontech (Mountain View, CA), according to the manufacturer’s instructions. FHL-2 (full length) cDNAs were labeled with [32P] dCTP using the RadPrime DNA Labeling System (Invitrogen). Unincorporated nucleotides were removed by spin column chromatography (Sephadex G-50; Amersham Biosciences). Loading conditions were determined by reprobing with GAPDH.

**Western blotting and immunoprecipitation**

Western blotting was performed as previously described. Briefly, cells extracts were prepared with lysis buffer (50 mmol/L Tris-Cl, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L NaF, 1 % Triton X-100, 1 mmol/L PMSF, 2 mmol/L Na3VO4). Samples containing 10 µg protein were separated on 10% sodium dodecylsulfate polyacrylamide (SDS/PAGE) gels and transferred to PVDF membranes (Hybond ECL™, Amersham Life Science Inc., Arlington Heights, IL), and incubated with
antibody against FHL-2 (1:1000, MBL, Nagoya, Japan), SK1 (1:200, Abgent, San Diego, CA), phospho-Akt, Akt, phospho-ERK, ERK, phospho-eNOS, eNOS (1:1000, Cell Signaling, Danvers, MA) or β-actin (1:5000, Sigma, Saint Louis, MO) at 4°C overnight. The membranes were then washed and incubated with a 1:2000 dilution of mouse or rabbit IgG-conjugated horseradish peroxidase-conjugated antibody (Amersham Biosciences). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham Biosciences) with Hyperfilm™-MP (Amersham Biosciences).

For immunoprecipitation assay, lysates were obtained from HAEC or BAEC. The cells were stimulated with VEGF 50 ng/mL, and after adjustment of protein concentration, lysate were precleared for 60 min at 4°C. After immunoprecipitation with anti-FHL-2 antibody (MBL, Nagoya, Japan) or anti-HA (Sigma) or anti-M2 Flag (Sigma) or anti-phosphotyrosine 4G10 (Millipore), the interaction between FHL-2 and SK1 was evaluated by immunoblotting with anti-M2 flag antibody (1:1000, Sigma), anti-HA antibody (1:1000 Sigma), anti-SK1 antibody (1:500 Abgent), and anti-FHL-2 (1:1000, MBL), or the phosphorylation of VEGFR2 was evaluated by immunoblotting with anti-VEGFR2 antibody (1:1000, Cell Signaling Technology). The results of immunoblot analysis were quantified by densitometry using Image-J software (NIH).

**Immunochemical staining**

Cells on glass coverslips were fixed in 4% paraformaldehyde (PFA) for 15 min and permeabilized with 0.2% Triton X-100 for 2 min. After blocking in 5% skim milk, samples were incubated with anti-HA antibody (1:100, Roche) and anti-flag (1:100, Sigma) antibodies at 4°C overnight. Corresponding secondary antibodies were
labeled with AlexaFluor 488 or 546 (1:200, Molecular Probes).

Sections of mouse heart and aorta were prepared as previously described.² Briefly, 4-µm frozen sections were fixed in 4% PFA for 15 min and then immunostained as aforementioned. Primary antibody used for the immunochemical staining was anti-FHL-2 (1:100, Abcam) antibody at 4°C overnight. Corresponding secondary antibodies were labeled with peroxidase (1:1000, GE healthcare).

**SK1 activity measurement**

SK activity analysis was performed as previously described.⁴ Briefly, cells were washed with chilled PBS and homogenized in chilled 0.1 mol/L phosphate buffer (pH 7.4) containing 20% glycerol, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 20 µmol/L ZnCl₂, 1 mmol/L Na₃VO₄, 15 mmol/L NaF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L 4-deoxypyridoxine. After ultracentrifugation at 100,000 g for 30 min, lysates were assayed for SK1 activity in reaction buffer (50 mmol/L HEPES [pH 7.4], 0.5% triton X-100, 15 mmol/L MgCl₂, 10% glycerol, 10 mmol/L NaF, and 1.5 mmol/L semicarbazide), based on the SK-catalyzed transfer of the γ-phosphate group of ATP using a mixture of cold ATP and [γ³²-P]ATP [1 mCi/sample] to a specific substrate, and the products were separated by TLC on Silica Gel G60 (Whatman, Maidstone, UK) using chloroform/methanol/acetic acid/water (90:90:15:6). The radioactive spots corresponding to sphingosine-1-phosphate were scraped and counted in a scintillation counter. In the SK reaction buffer used which contained 0.5% Triton X-100, SK2 activity has been shown to be inhibited by ∼96%.⁵

**Phosphatidylinositol 3-kinase assay**
The measurement of phosphatidylinositol 3-kinase activity was performed as previously described. Briefly, cell lysates were incubated with antibody against the p85 or p110 subunit (Upstate Biotechnology, Inc. Lake Placid, NY). The washed pellets were washed and resuspended in 50 ml of kinase reaction buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.5 mmol/L EGTA) and incubated at 25 °C for 10 min after the addition of 0.5 μl of 20 mg/ml phosphatidylinositol dissolved in chloroform to make micelles of PI. Assay were initiated with the addition of 5 μl of [γ-32P] ATP solution and incubated at room temperature for 30 min. During this time, the formation of phosphatidylinositol phosphate was linear. The reaction was stopped after the addition of 100 μl of chloroform, methanol, 11.6 mol/L HCl (100:200:2). After centrifugation, the lower organic phase was taken for thin layer chromatography (TLC) on Silica Gel plates (Whatman, Maidstone, UK) to be developed in chloroform, methanol, 25% ammonium hydroxide, water (43: 38: 5: 7). The plates were exposed to film, and radiolabeled spots were identified.

[^35S] GTPγS binding assay

The measurement of[^35S] GTPγS binding was performed as previously described, which measures GDP-GTP exchange on the Gα subunit by coupling of S1P receptors and G proteins. Cells were washed with cold PBS, suspended in 20 mmol/L HEPES, pH 7.4, 10 mmol/L EDTA and protease inhibitor (Roche). The homogenates were centrifuged at 40,000 g for 30 min at 4 °C, and the membranes were solubilized at 4 °C in buffer containing 20 mmol/L HEPES, pH7.4. The solubilized membranes were incubated at 37 °C in a medium containing 100 mmol/L[^35S]GTPγS and 10 mmol/L HEPES, pH7.4. Ten volumes of 100 mmol/L Tris-HCl, pH8.0 containing 10 mmol/L MgCl2, 100 mmol/L NaCl and 20 μmol/L GTP were used to stop the reaction.
Membrane proteins were solubilized with 1% Nonidet P-40 under nondenaturing conditions, and Goi subunits were immunoprecipitated using anti-Goi antibody (sc-28586, Santa Cruz). Bound radioactivity was quantitated by scintillation spectrometry and described as fold increase.

**c-fos promoter assay and MTS assay**

Proliferation rate of BAEC was determined using a c-fos-dependent promoter luciferase activity assay as previously described.¹ BAEC were plated on 24-well plate (1×10⁵ cells/well) and incubated for 24 h. Next, 80-90% confluent BAEC were transfected with FHL-2 or GFP and a c-fos-dependent promoter-luciferase gene construct and incubated for 24 h in DMEM containing 10% FCS. After incubation in starvation medium over night, BAEC were treated with or without VEGF 50 ng/mL for 24 h. At 24 h after treatment, cells were assayed for luciferase activity according to manufacturer’s protocol (Luciferase Assay System, Promega, Madison, WI).

The effect of FHL-2 overexpression or knockdown on BAEC viability was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madiom, WI) as previously described.⁹ BAEC were seeded onto 24-well plates (1×10⁵ cells/well). After cells were grown to 40-50% confluency, BAEC were incubated in DMEM containing 10% FCS overnight. After incubation in starvation medium over night, 200 μL of fresh medium containing MTS reagent was pipetted into each well. After 3-4 h incubation, the absorbance of the supernatant from each well was measured at 490 nm.

**Cell migration assay**

Migration of BAEC or HAEC transfected with FHL-2 plasmid or GFP plasmid
was measured using a modified Boyden chamber assay as previously described. Briefly, serum starved-BAEC were treated with or without VEGF 100 ng/mL for 8 h. After incubation, cell suspension (50 μL, 6-8×10^5 cells) was added to the upper chamber and incubated for 4 h at 37°C and in a 5% CO₂ atmosphere. The non-migrating cells in the upper chamber were scraped off, and washed with PBS. The migrated cells were fixed, stained (DiffQuick, Sysmex, Kobe, Japan), photographed, and counted using microscopy.

Cell migration was also examined by scratch wound assay. BAEC were plated on 6-well plate (1×10^5 cells/well) and incubated for 24 h. 80-90% confluent BAEC were transfected with FHL-2 or GFP gene construct and incubated for 24 h in DMEM containing 10% FCS. After incubation in starvation medium over night, BAEC were treated with or without VEGF 50 ng/mL or S1P 10 μmol/L, followed one day later by wounding of the confluent monolayer by scraping a pipette tip across. Cells were photographed using microscopy.

**RT-PCR analysis**

Total RNA was extracted from Xenopus embryo at stage 32-33 with Isogen (Nippongene, Tokyo, Japan) according to manufacturer’s instruction. cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen). PCRs were performed using the following primers:

ODC (ornithine decarboxylase),
forward 5’-GTCAATGATGGAGTGTATGGATC-3’
reverse 5’-TCCATTCCGCTCTCCTGAGCAC-3’

FHL-2,
forward 5’-GAAGCCTGTTTCCACTGCTC-3’
reverse 5’-TTTGGGGATGAAACTCTTGG-3’
SK1,
forward 5’-GCTGATGTGGACCTAGAGAG-3’
reverse 5’-CTTCCTACAGGGAGGTAAGC-3’

Real-time quantitative PCR analysis

Total RNAs were prepared from HAEC using RNeasy (Qiagen, Stanford, CA), and cDNA were synthesized by the Thermo Script RT-PCR System (Invitrogen, Carlsbad, CA). Relative mRNA levels of FHL-2 and SK1 were quantified by real-time RT-PCR using the TaqMan Gene Expression Assay (human FHL-2: Hs00179935, human SK1: Hs00184211, human β-actin: Hs99999903, Applied Biosystems, Foster City, CA). The absolute number of gene copies was normalized against β-actin and was standardized to a sample standard curve.

In vivo Xenopus embryo RNA injection model

Xenopus laevis embryos were generated using standard techniques and staged as previously described.11 FHL-2 cDNA and GFP cDNA were cloned into pCS2+. Synthetic RNA was made from linearized plasmid DNA with mMMESSAGE mMACHINE™ (Ambion, Austin, TX). For blastomere injections, 3000 pg of synthetic mRNA encoding FHL-2 or GFP was microinjected into one of the vegetal-dorsal blastomeres at the eight-cell stage. After overnight incubation with 3% Ficoll in Steinberg’s solution at 20°C, embryos were transferred to Steinberg’s solution and collected at stage 32-33. Whole mount in situ hybridization was performed as described previously described,12 using the DIG RNA Labeling kit (Roche, Basel, Switzerland).
Positive signals were visualized using BM purple (Roche). To evaluate vascular formation of Xenopus embryos, embryos were hybridized with probes for EC marker, Xenopus fli-1 or msr.\textsuperscript{15}

**Statistical analysis**

All values are expressed as mean ± SD. Analysis of variance with subsequent Fisher’s PLSD test or the unpaired Student’s $t$ test was employed to determine the significance of differences in multiple comparisons. All statistical analysis was performed using Stat-View 5.0 software (SAS. Institute, Inc., NC). Values of $p<0.05$ were considered to represent statistical significance.

**Supplement Figure Legend**

**Supplementary Figure I.** Expression of FHL-2 in heart and vascular EC. A, Northern blot of FHL-2 mRNA in human cardiovascular tissue. Upper panel shows that human FHL-2 gene is expressed in all compartment of the heart, including the aorta. Lower panel shows GAPDH expression, used as loading control. B, Expression of FHL-2 in heart (upper panel) and ascending aorta (lower panel). Representative cross sections by DAB immunohistochemical staining (x 200 magnification). “FHL-2” indicates staining with anti-FHL-2 antibody. “NC” indicates negative control. C, Expression of FHL-2 in EC, SMC and macrophages. “β-actin” indicates loading control. D, Expression of FHL-2 in HAEC at 24 h after stimulation with various angiogenic factors. “VEGF” indicates human recombinant VEGF 25 ng/mL. “FGF2” indicates human recombinant FGF2 25 ng/mL. “EGF” indicates human recombinant EGF 25 ng/mL.
“VEGF+FGF2+EGF” indicates mixture of human recombinant VEGF 25 ng/mL, FGF2 25 ng/mL and EGF 25 ng/mL. “TNF-α” indicates human recombinant TNF-α 10 ng/mL. “β-actin” indicates loading control.

**Supplementary figure II.** FHL-2 suppresses VEGF-induced proliferation in BAEC or HAEC. A, MTS assay in BAEC, n=4. B, c-fos promoter assay in BAEC, n=8. C, MTS assay in HAEC, n=4. “control” indicates transfection with GFP gene. “FHL-2” indicates transfection with FHL-2 gene. “VEGF” indicates treatment with human recombinant VEGF 50 ng/mL for 24 h. #P< 0.01 vs. control.

**Supplementary figure III.** A, Immunoprecipitation analysis of overexpressed HA-FHL-2 and Flag-SK1 with or without VEGF treatment. “IP: HA-FHL-2” indicates immunoprecipitation with anti-HA antibody. “IP: Flag-SK1” indicates immunoprecipitation with Flag antibody. “IP: control IgG” indicates immunoprecipitation with normal mouse IgG. “VEGF” indicates treatment with human recombinant VEGF 50 ng/mL for 5 min. “IB: Flag-SK1” indicates immunoblot with anti-Flag antibody. “IB: HA-FHL-2” indicates immunoblot with anti-HA antibody. B. Quantification of FHL-2 and SK1 mRNA by real time PCR in HAEC treated with VEGF 50 ng/mL for 0, 6, 12, and 24 h. C, Representative western blot of FHL-2 and SK1 in HAEC treated with VEGF 50 ng/mL for 0, 6, 12 and 24 h. Expression of β-actin was used as loading control.

**Supplementary Figure IV.** A, Western blot of phosphorylated Akt and total Akt in HAEC at 0, 10, 20 and 30 min after VEGF treatment with or without SK1 inhibitor,
DMS. B, Western blot of phosphorylated Akt and total Akt in HAEC at 0, 10, 20, and 30 min after VEGF treatment with or without S1P receptors antagonist, VPC23019. C, Migration assay by modified Boyden chamber method in HAEC. Upper graph shows the number of cells per field and lower panels show the representative pictures (x 200 magnification). D, Migration assay by modified Boyden chamber method in HAEC. Upper graph shows the number of cells per field and lower panels show the representative pictures (x 200 magnification). “VEGF” indicates treatment with human recombinant VEGF 50 ng/mL. “VEGF+DMS” indicates treatment with human recombinant VEGF 50 ng/mL in the presence of DMS (dimethylshingosine; 10 μmol/L), SK-1 inhibitor. “S1P” indicates treatment with sphingosine-1-phosphate 10 μmol/L. “S1P+DMS” indicates treatment with sphingosine-1-phosphate 10 μmol/L in the presence of DMS 10 μmol/L. “VEGF+VPC23019” indicates treatment with human recombinant VEGF 50 ng/mL in the presence of S1P receptors antagonist, VPC23019 (10 μmol/L). #P<0.0001 vs. no treatment. n=3 .E, Western blotting of phosphorylation of Akt in BAEC in time course at 0, 5, 10, 15 and 20 min after stimulation with VEGF 50 ng/mL. Upper panel shows the representative pictures and lower panel shows the densitometry analysis. “control” indicates overexpressed GFP gene, “FHL-2” indicates overexpressed FHL-2 gene. Corresponding Akt expression was used as loading control. n=3.

**Supplementary figure V.** A, Phosphatidylinositol 3-kinase activity by phosphatidylinositol-3,4,5-trisphosphate (PIP) production associated with p110 and p85 in BAEC in time course at 0, 10, 20 and 30 min after stimulation with VEGF 50 ng/mL or S1P 10 μmol/L . n=3. B, S1P receptors activation in BAEC was analyzed by [35S]
GTPγS binding assay in the presence or absence of overexpressed FHL-2 at 10 min after treatment of VEGF or S1P. #P<0.01 vs. no treatment. n=4. C, FHL-2 suppresses VEGF-induced migration in HAEC, but not S1P-induced migration. Modified Boyden chamber assay in HAEC. Upper panel shows the number of cells per field and lower panels show the representative pictures (x 200 magnification). D, Scratch wound assay in BAEC (x 100 magnification). “control” indicates overexpressed GFP gene, “FHL-2” indicates overexpressed FHL-2 gene. “VEGF” indicates treatment with human recombinant VEGF 50 ng/mL. “S1P” indicates treatment with sphingosine-1-phosphate 10 μmol/L. #P<0.0001 vs. control no treatment. *P<0.0001 vs. FHL-2 no treatment. n=3.

Supplementary figure VI. A, Extracted proteins from control or FHL-2 siRNA transfected BAEC were subjected to determine the most effective siRNA for function analysis after 48 h. “scramble siRNA” indicates transfection with scramble siRNA as control. “254 siRNA”, “439 siRNA” and “511 siRNA” indicate transfection with FHL-2 siRNA. “FHL-2” indicates immunoblot with anti-FHL-2 antibody. Expression of β-actin was used as loading control. B, DMS, SK1 inhibitor, does not affect VEGF-induced phosphorylation of VEGFR2. VEGFR2 activation was analyzed by immunoprecipitation analysis with anti-phosphotyrosine antibody (4G10) and anti-VEGFR2 antibody after VEGF treatment with or without DMS. C, VEGFR2 activation was analyzed by immunoprecipitation analysis with anti-phosphotyrosine antibody (4G10) and anti-VEGFR2 antibody after S1P treatment in the absence or presence of overexpressed FHL-2 or FHL-2 siRNA. “IP: 4G10” indicates immunoprecipitation with anti-phosphotyrosine antibody. “IB: VEGFR2” indicates
immunoblot with anti-VEGFR2 antibody. “in put” indicates loaded cell lysate. “IP: control IgG” indicates immunoprecipitation with normal mouse IgG. “Lysate” indicates immunoblot with anti-VEGFR2 antibody of total protein. “VEGF” indicates treatment with VEGF 50 ng/mL. “S1P” indicates treatment with sphingosine-1-phosphate 10 μmol/L. D, Knockdown of FHL-2 by siRNA enhanced VEGF-induced phosphorylation of Akt and ERK by western blotting. Analysis of phosphorylation of Akt and ERK by western blot was performed in BAEC after treatment with VEGF for 0, 5, 10, 20 and 30 min. Total Akt, ERK and β-actin expression was used as loading control. “scramble siRNA” indicates transfection with scramble siRNA against FHL-2. “FHL-2 siRNA” indicates transfection with siRNA 439 against FHL-2. E and F, S1P receptors activation by [35S] GTPγS binding assay and phosphatidylinositol 3-kinase activity by phosphatidylinositol-3,4,5-trisphosphate (PIP) production associated with p110 and p85 in the absence and presence of FHL-2 siRNA with treatment of S1P receptor antagonist, VPC23019 (10 μmol/L) in BAEC. n=3.

**Supplementary figure VII.** Expression of FHL-2 and SK1 were analyzed by RT-PCR of total RNA prepared from Xenopus embryo at stage 32-33 in template (+). As a loading control, the same samples were analyzed for ornithine decarboxylase (ODC) mRNA expression. “template (RT-)” indicates PCR with no template control.
Reference


