FGD5 Mediates Proangiogenic Action of Vascular Endothelial Growth Factor in Human Vascular Endothelial Cells

Yusuke Kurogane, Muneaki Miyata, Yoshiki Kubo, Yuichi Nagamatsu, Ramendra K. Kundu, Akiyoshi Uemura, Tatsuro Ishida, Thomas Quertermous, Ken-ichi Hirata, Yoshiyuki Rikitake

Objective—Vascular endothelial growth factor (VEGF) exerts proangiogenic action and induces activation of a variety of proangiogenic signaling pathways, including the Rho family small G proteins. However, regulators of the Rho family small G proteins in vascular endothelial cells (ECs) are poorly understood. Here we attempted to clarify the expression, subcellular localization, downstream effectors, and proangiogenic role of FGD5, a member of the FGD family of guanine nucleotide exchange factors.

Methods and Results—FGD5 was shown to be selectively expressed in cultured human vascular ECs. Immunofluorescence microscopy showed that the signal for FGD5 was observed at peripheral membrane ruffles and perinuclear regions in human umbilical vein ECs. Overexpression of FGD5 increased Cdc42 activity, whereas knockdown of FGD5 by small interfering RNAs inhibited the VEGF-induced activation of Cdc42 and extracellular signal–regulated kinase. VEGF-promoted capillary-like network formation, permeability, directional movement, and proliferation of human umbilical vein ECs and the reorientation of the Golgi complex during directional cell movement were attenuated by knockdown of FGD5.

Conclusion—This study provides the first demonstration of expression, subcellular localization, and function of FGD5 in vascular ECs. The results suggest that FGD5 regulates proangiogenic action of VEGF in vascular ECs, including network formation, permeability, directional movement, and proliferation. (Arterioscler Thromb Vasc Biol. 2012;32: 988-996.)

Key Words: angiogenesis ■ endothelial function ■ signal transduction ■ GEF ■ small G proteins

Angiogenesis plays an important role in a number of physiological and pathological processes. Angiogenesis is promoted by the action of proangiogenic growth factors, and vascular endothelial growth factor (VEGF) is a major proangiogenic agent that regulates multiple key steps of angiogenesis. VEGF exerts its proangiogenic action by binding to VEGF receptor 2, which triggers activation of a variety of signaling molecules, such as phosphatidylinositol 3-kinase, Akt, extracellular signal–regulated kinase (ERK), and the Rho family small G proteins. The Rho family consists of Rho, Rac, and Cdc42, which are involved in the reorganization of the actin cytoskeleton and known to be critical for morphological changes during cell movement and differentiation. It is known that Rho regulates actin stress fiber formation, whereas Rac and Cdc42 regulate the formation of protrusions, such as lamellipodia and filopodia. There is a growing body of evidence that the Rho family small G proteins play important roles in the cardiovascular system. VEGF regulates endothelial cell (EC) movement and vascular permeability in which the activities of the Rho family small G proteins are dynamically changing. The regulators of Rho, Rac, and Cdc42 activities in VEGF-treated vascular ECs are poorly understood, although it was reported that Vav2, a guanine nucleotide exchange factor (GEF) for Rac, is involved in the VEGF-induced activation of Rac1.

Small G proteins cycle between the GDP-bound inactive form and the GTP-bound active form. The exchange of GDP for GTP is facilitated by GEFs. On the other hand, inactivation occurs through the intrinsic GTPase activity, which converts bound GTP into GDP and is stimulated by regulatory factors called GTPase activating proteins. In this way, 1 cycle of activation and inactivation is achieved, and small G
proteins serve as biomarkers that regulate downstream biological responses by controlling the reaction period to transduce upstream signals to downstream effectors. To date, no endothelial-specific GEF or GTPase activating protein has been reported.

In a virtual subtraction screening to identify novel molecules whose expression is restricted to ECs, FGD5 was identified as a candidate molecule. FGD5 is a member of the FGD GEF family consisting of FGD1, FGD2, FGD3, FGD4/frabin, FGD5, FGD6, and FRG. FGD5 comprises, in order, a glutamate-rich N-terminal region, a Dbl-homology (DH) domain, a first pleckstrin homology (PH) domain adjacent to the DH domain, an FYVE domain, and a second C-terminal PH domain. Although FGD1–4 and FRG have been characterized to be a GEF for Cdc42, FGD5 and FGD6 have not been characterized, and their target small G proteins are not known. Here we investigate the expression of FGD5 in vascular ECs and identify the target small G protein and proangiogenic functions of this protein.

Methods

Antibodies and Reagents
Mouse anti-FGD5 polyclonal antibody (pAb) (Abnova), rabbit anti-FGD5 pAb (Sigma–Aldrich), mouse anti-actin monoclonal antibody (mAb), rabbit anti-Cdc42 pAb (Santa Cruz Biotechnology), rabbit anti-myec tag pAb, rabbit anti-phospho-ERK1/2 pAb, rabbit anti-Erk pAb, rabbit anti-phospho-Akt (Ser473) pAb, rabbit anti-Akt pAb (Cell Signaling Technology), fluorescein isothiocyanate (FITC)–phalloidin (Invitrogen), mouse anti-calnexin mAb (Abcam), mouse anti-GM130 mAb, mouse anti-EEA1 mAb, mouse anti-vascular endothelial-cadherin mAb (BD Transduction Laboratories), and 4′,6-diamidino-2-phenylindole (Invitrogen) were purchased from commercial sources. Horseradish peroxidase–conjugated secondary antibodies were purchased from GE Healthcare Bioscience. Fluorophore (FITC and Cy3)-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Human recombinant VEGF and fibroblast growth factor-2 were purchased from PeproTech.

Plasmids
Human cDNA including the coding region (amino acids 242–1462) of FGD5 (FLJ00274) was purchased from Kazusa DNA Research Institute. cDNA for an N-terminal fragment of FGD5 (amino acids 1–241) was obtained by reverse transcription–polymerase chain reaction (RT-PCR) using human umbilical vein endothelial cell (HUVEC) cDNA as a template. The full-length cDNA for human FGD5 was constructed by cDNAs for the N-terminal fragment and the C-terminal region (amino acids 242–1462) and subcloned into pFLAG-CMV2b (pFLAG-CMV2b-FGD5). cDNA encoding the DH/PH region (amino acids 887–1223) was obtained by RT-PCR and subcloned into pFLAG-CMV2b (pFLAG-CMV2b-FGD5 (DH/PH)).

RT-PCR
RNAs were extracted from mouse organs or cultured cells using TRizol (Invitrogen). cDNA was synthesized by SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed by standard methods using the following primers: mouse FGD5 forward, GACATCTGTAACACACGACACTG; reverse, ACTCTGCTAAAGGAATGGTTTATT; human FGD5 forward, TACAGGTTTGGGATGACACGAGG; reverse, CCGGGCAATGTTGCTCTG; mouse GAPDH forward, GAACCCCTTATGTGCCTCTCA; reverse, TTCTTACTCCTCGAGGCCTAGT; human GAPDH forward, CTGATGCCCCATGTCTGC; reverse, CACCCCTTGTGCTAGCACAATTG.

Role of FGD5 in VEGF-Induced Angiogenesis

In Situ Hybridization
Whole-mount in situ hybridization of mouse embryos at embryonic day 10 was performed as described. Briefly, digoxigenin-labeled probes were transcribed from linearized cDNA template and applied to embryos digested with proteinase K. Embryos were incubated with BM purple substrate, and images were taken with a charged-coupled device digital camera. In situ hybridization and immunofluorescence using rabbit anti-type IV collagen pAb (Cosmo Bio Co) in whole-mount retinas of postnatal day 4 and postnatal day 8 mice were performed as described.

Cell Culture and Transfection Experiment
Primary cultures of HUVECs were obtained from Lonza and maintained at 37°C using the EGM-2 bullet kit (Lonza). Cells between passages 2 and 6 were used in each experiment. For small interfering RNA (siRNA) experiments, cells were transfected with Stealth RNAs for FGD5 or Cdc42, or nonsilencing negative control (Invitrogen) using Lipofectamine RNAmax (Invitrogen) according to the manufacturer’s instructions. Forty-eight or 72 hours after transfection, cells were subjected to each experiment.

Western Blotting and Pull-Down Assay
Western blotting and pull-down assay using GST-PAK-CRIB were performed as described previously.

Assays for Capillary-Like Network Formation, Wound Healing, Polarization of the Golgi Complex, Migration, Apoptosis, Proliferation, and Immunofluorescence Microscopy
Capillary-like network formation on Matrigel, a wound healing assay, an assay for polarization of the Golgi complex, a Boyden chamber assay, an apoptosis assay, a cell proliferation assay, and immunofluorescence microscopy were performed as described previously.

Permeability Assay
A permeability assay was performed as described previously. In brief, HUVECs, transfected with control or FGD5 siRNAs, were seeded onto gelatin-coated, 6.5-mm Transwell 0.4-μm pore size culture inserts (in 24-well culture dishes, Corning Life Sciences) at a density of 1×10⁴ cells/well and cultured for 72 hours to form restrictive endothelial monolayers. After cells were starved overnight with EB2M-2 (Lonza) supplemented with 0.5% fetal bovine serum (starvation medium), FITC-dextran (70 kDa) (Research Organics) dissolved in the starvation medium was placed in the apical compartment at a final concentration of 500 μg/mL. To determine the baseline flux of FITC-dextran, samples (10 μL) were taken from the basolateral compartment at 0, 30, and 60 minutes. Flux was calculated as the slope of the best-fit line of accumulated FITC-dextran versus time, using the least-squares method. After the 60-minute baseline period, 200 ng/mL VEGF was added to the apical chamber, and samples (10 μL) were taken from basolateral compartment at 90 and 120 minutes to determine FITC-dextran flux. Diffusible FITC dextran in the basolateral compartment medium was measured with a fluorometer (excitation, 485 nm; emission, 538 nm).

Results
Expression of FGD5 in Mouse Organs and Cultured Human Cells
We examined mRNA expression of FGD5 in mouse organs and cultured cells by RT-PCR. FGD5 mRNA was detected in various highly vascular mouse organs, including the lung, kidney, and ovary (Figure 1A). FGD5 mRNA was detected only in human ECs among the various cell types examined (Figure 1B). Western blotting using the anti-FGD5 antibody revealed the protein expression of FGD5 in human
ECs (Figure 1C). Thus, FGD5 appears to be predominantly expressed by ECs.

To determine the expression pattern of FGD5, we performed whole mount in situ hybridization experiments on staged mouse embryo. At embryonic day 10, FGD5 transcript was detected in the intersomitic vessels, dorsal aorta, and brain vasculature (Figure 1D–1F), with an expression pattern identical to that of an endothelial marker AA4,17 demonstrating a vascular expression pattern of FGD5. The expression of FGD5 in mouse retina was analyzed. FGD5 transcript was strongly detected in the developing retinal vasculature at postnatal day 4, whereas its expression was downregulated in preformed vessels at postnatal day 8 (Figure 1G). Compared with arteries, FGD5 transcript was predominantly detected in capillaries and veins, and particularly at the advancing vascular fronts.

We examined intracellular localization of FGD5 in HUVECs by immunofluorescence microscopy. The signal for FGD5 was observed in peripheral membrane ruffles and in perinuclear regions (Figure 2A–2D). In peripheral membrane ruffles, the signal for FGD5 was merged with the signal for F-actin (Figure 2A). In the perinuclear regions, the signal for FGD5 was merged, in part, with the signals for the endoplasmic reticulum marker calnexin, the Golgi marker GM130, and the early endosome marker EEA1 (Figure 2B–2D).

Regulation of the VEGF-Induced Activation of Cdc42 by FGD5

Because other members of the FGD family GEFs are known to activate Cdc42,7–11 we investigated whether FGD5 could induce the activation of Cdc42. Transfection of full-length FGD5 increased Cdc42 activity compared with transfection of the vector alone, as assessed by the pull-down assay, indicating the activation of Cdc42 by FGD5 (Figure 3A). The DH/PH domain is known to be the functional GEF domain of...
many Rho-GEFs, including FGD1 and FRG. Transfection of FRG (DH/PH), the DH/PH domain of FRG, increased Cdc42 activity, whereas transfection of FGD5 (DH/PH) failed to induce the activation of Cdc42 (Figure 3A). Transfection of full-length FGD5 did not induce the activation of TC10 (RhoQ) or TCL (RhoJ), members of the Rho family small G proteins closely related to Cdc42 (Figure 3B and 3C). To further examine whether FGD5 is involved in the VEGF-induced activation of Cdc42, FGD5 was knocked down by siRNA. FGD5 was effectively knocked down by 3 different specific siRNAs (Figure 3D). VEGF induced the activation of Cdc42, and the VEGF-induced activation of Cdc42 was significantly inhibited by knockdown of FGD5 (Figure 3E). Thus, FGD5 regulates the VEGF-induced activation of Cdc42.

Figure 2. Intracellular localization of FGD5 in human umbilical vein endothelial cells (HUVECs). HUVECs were left untreated (-) or directionally stimulated by vascular endothelial growth factor (VEGF) and then stained doubly with the anti-FGD5 antibody and phalloidin (A), the anti-calnexin monoclonal antibody (mAb) (B), the anti-GM130 mAb (C), or the anti-EEA1 mAb (D). The results shown are representative of 3 independent experiments, and identical results were obtained. After stimulation with VEGF, FGD5 was observed in peripheral membrane ruffles, and merged with the signal for F-actin (arrows). Scale bars = 20 μm.

Regulation of the VEGF-Induced In Vitro Angiogenesis by FGD5
We investigated the role of FGD5 in the VEGF-induced in vitro angiogenesis by comparing proangiogenic responses between FGD5-knockdown and control siRNA-transfected HUVECs. VEGF promoted capillary-like network formation of control siRNA-transfected HUVECs on Matrigel (Figure 4A). However, capillary-like network formation was significantly attenuated in FGD5-knockdown HUVECs (Figure 4A and 4B).

Immunofluorescence microscopy showed that the signal for FGD5 was concentrated at the cell-cell contact sites between adjacent HUVECs and was colocalized with the signal for vascular endothelial-cadherin (Figure 4C). The signal for FGD5 at the cell-cell contact sites was markedly decreased in FGD5-knockdown cells. We then compared vascular permeability between FGD5-knockdown and control siRNA-transfected HUVECs using a nonionic solute, FITC-conjugated dextran. In control siRNA-transfected cells, permeability of HUVEC monolayer was significantly increased by 1.3-fold in the presence of VEGF, whereas in FGD5-knockdown HUVECs, permeability was similar to that in control siRNA-transfected cells in the absence of VEGF and was not increased by VEGF (Figure 4D). Thus, FGD5 plays a role in the VEGF-induced permeability.

Compared with control siRNA-transfected cells, wound closure was delayed in FGD5-knockdown HUVECs regardless of the presence or absence of VEGF treatment, indicating that directional cell movement was inhibited by knockdown of FGD5 (Figure 5A and 5B). The alignment of the Golgi complex in cells at the wound edge was analyzed because the reorientation of the Golgi complex in moving cells has been reported to correlate with directional cell movement. The frequency of the Golgi complex facing the wound was decreased in FGD5-knockdown cells compared with control siRNA-transfected cells (Figure 5C and 5D). Directional cell migration was also analyzed by the Boyden chamber assay. VEGF increased migration of control siRNA-transfected HUVECs, whereas VEGF failed to increase migration of FGD5-knockdown HUVECs (Figure 5E). These results indicate that FGD5 regulates activity and directionally of cell movement.
The annexin V staining assay demonstrated that apoptosis was not enhanced by knockdown of FGD5 (Figure 6A). However, VEGF stimulated proliferation of HUVECs, and the VEGF-induced proliferation was inhibited by knockdown of FGD5 (Figure 6B). Similarly, other proangiogenic agent fibroblast growth factor-2 stimulated proliferation of HUVECs and the fibroblast growth factor-2–induced proliferation was inhibited by knockdown of FGD5. However, the fetal bovine serum–induced proliferation was not attenuated by knockdown of FGD5 (Figure I in the online-only Data Supplement). Because the VEGF-induced proliferation of vascular ECs is regulated by ERK activity, the role of FGD5 in the regulation of ERK activity was examined. VEGF increased ERK phosphorylation and the VEGF-induced increase in ERK phosphorylation was significantly attenuated by knockdown of FGD5 (Figure 6C). We then examined the role of FGD5 in Akt activity, which regulates EC survival. Knockdown of FGD5 had no effects on Akt phosphorylation (Figure II in the online-only Data Supplement), consistent with its effect on apoptosis. To examine whether Cdc42 is involved in the FGD5-mediated ERK phosphorylation, Cdc42 was knocked down by siRNA. Cdc42 was effectively knocked down by 3 different specific siRNAs (Figure 6D). The VEGF-induced increase in ERK phosphorylation was not attenuated by knockdown of Cdc42 (Figure 6E). This result indicates that VEGF induces ERK activation in an FGD5-dependent but Cdc42-independent manner. Collectively, these results indicate a critical role of FGD5 in the proangiogenic action of VEGF, and possibly the proangiogenic action of fibroblast growth factor-2.

**Discussion**

In the present study, we demonstrated that FGD5 was selectively expressed in cultured human vascular ECs and that knockdown of FGD5 in HUVECs inhibited the VEGF-induced capillary-like network formation, permeability, directional movement, and proliferation. It was recently reported that in zebrafish the FGD5 gene is specifically expressed in vascular ECs and induced by a transcription factor, Etsrp, that is required for vasculogenesis and primitive myelopoiesis. Our results showing the EC-restricted expression pattern of FGD5 are consistent with
the findings in zebrafish, although the role of FGD5 in vascular development has not been elucidated.

FGD proteins comprise a family consisting of FGD1 to FGD6 and FRG. FGD1 expression is restricted to skeletal tissue, and FGD1 has been implicated in skeletal development, with mutations in the FGD1 gene leading to facio-genital dysplasia (Aarskog-Scott syndrome), an X-linked developmental disorder characterized by a disproportionately short stature and by facial, skeletal, cardiac, ocular, and urogenital anomalies. FGD2 is expressed in antigen-presenting cells, such as B lymphocytes, macrophages, and dendritic cells. FGD3 is expressed in skeletal muscles. FGD4/frabin is ubiquitously expressed, and mutations of FGD4 gene have been implicated in Charcot-Marie-Tooth 4H subtype, an autosomal recessive neuropathy characterized by muscle weakness and wasting, foot and hand deformities, and electrophysiological changes. Thus, FGD1 to FGD3 exhibit a relatively tissue-restricted expression pattern, suggesting that a specified member of the FGD protein family functions in different tissues and cells, and therefore mutations or abnormalities of FGD proteins are related tightly to human diseases. Future studies to investigate whether FGD5 is associated with vascular diseases or developmental abnormality are needed.

Figure 4. Regulation of the vascular endothelial growth factor (VEGF)-induced capillary-like network formation and permeability by FGD5. A and B, Inhibition of capillary-like network formation by knockdown of FGD5. Human umbilical vein endothelial cells (HUVECs), transfected with control or FGD5 small interfering RNAs (siRNAs), were seeded onto Matrigel and incubated for 16 hours. Representative images (A) and quantitative results (B) are shown. The results shown are the means±SE of 4 independent experiments. C, Localization of FGD5 in a HUVEC monolayer. A monolayer of HUVECs, transfected with control or FGD5 siRNAs, was stained doubly with the anti-FGD5 antibody and the anti-vascular endothelial (VE)-cadherin monoclonal antibody. The results shown are representative of 3 independent experiments, and identical results were obtained. Scale bars=20 μm. D, Inhibition of HUVEC monolayer permeability by knockdown of FGD5. The results shown are the means±SE of 4 independent experiments. †p<0.01. NS indicates not significant.
migration of ECs and therefore be required for angiogenesis. Importantly, the FGD5 target Cdc42 is activated by VEGF, and its activation triggers filopodia formation and regulates cell polarization through microtubule organization. A recent study demonstrated a role for Cdc42 in the VEGF-promoted angiogenesis. Cdc42 improved the coordination between actin filaments and microtubules and enhanced the formation of vascular cords, suggesting that Cdc42 rectifies defects in angiogenesis by improving cytoskeletal dynamics and capillary morphogenesis. Moreover, it was reported that Cdc42 induces lumen and vacuole formation in ECs. In addition, we observed partial overlapping of cellular localization of FGD5 with that of the early endosome marker EEA1. A similar localization pattern was observed for FGD2 that is localized to early endosomes and membrane ruffles. However, the biological significance of the endosomal localization of FGD5 remains unknown. Cdc42 has a fundamental role in the coordination of membrane transport events via the reorganization of the actin cytoskeleton and other signaling events. Therefore, FGD5 localized to early endosomes might be involved in the control of membrane transport via the local activation of Cdc42. In addition, FGD5 localized to membrane ruffles presumably regulates cell migration, as FGD2 and FGD4 are localized to membrane ruffles. It was reported that active ERK locates at membrane ruffles and regulates their formation. Therefore, it is possible that FGD5 may regulate membrane ruffle formation through ERK activation.

We showed here that despite the almost complete silencing of FGD5 expression, the VEGF-induced activation of Cdc42 was not completely inhibited by knockdown of FGD5, implying that other Cdc42-GEFs are involved. In summary, our findings suggest a novel role for FGD5 in VEGF-induced angiogenesis through Cdc42 activation.

Figure 5. Regulation of the vascular endothelial growth factor (VEGF)-induced directional cell movement by knockdown of FGD5. A and B, Inhibition of directional cell movement by knockdown of FGD5. Human umbilical vein endothelial cells (HUVECs), transfected with control or FGD5 small interfering RNAs (siRNAs), were subjected to the wound healing assays. Representative images (A) and quantitative results (B) are shown. The quantitative results shown are the means±SE of 5 independent experiments. C and D, Inhibition of the reorientation of the Golgi complex by knockdown of FGD5. HUVECs, transfected with control or FGD5 siRNAs, were subjected to the wound healing assays and stained with the anti-GM130 monoclonal antibody, phalloidin, and 4’,6-diamidino-2-phenylindole (DAPI). Representative images (C) and quantitative results (D) are shown. The quantitative results shown are the means±SE of 5 independent experiments, in which at least 6 randomly chosen fields were analyzed in each experiment. E, Inhibition of directional cell movement by knockdown of FGD5. HUVECs, transfected with control or FGD5 siRNAs, were subjected to the Boyden chamber assays. The quantitative results shown are the means±SE of 4 independent experiments. †P<0.01. Scale bars=50 μm.
that of FGD4 does not induce the formation of filopodia and lamellipodia. These findings suggest that additional domains are necessary for the activation of Cdc42. Future studies are required to clarify a mechanism of the activation of Cdc42 by FGD5.

We showed that the VEGF-induced proliferation of HUVECs and phosphorylation of ERK was attenuated by knockdown of FGD5. Because ERK activity is critical for the VEGF-induced proliferation of vascular ECs,21–23 the decreased phosphorylation of ERK appears to contribute to the impaired proliferation in FGD5-knockdown cells. Our finding that FGD5 is located upstream of ERK is consistent with previously published results demonstrating that FGD1 mediates the Ras-induced activation of ERK through the activation of the Cdc42-p21-activated kinase 1-MAPK/ERK kinase-ERK signaling pathway.35 However, ERK phosphorylation was not inhibited by knockdown of Cdc42. This result suggests the possibility that FGD5 regulates ERK activity independently of its capacity to activate Cdc42. Future studies are needed to clarify the signaling mechanism underlying FGD5-mediated ERK activation.

In conclusion, FGD5 regulates Cdc42 activity and mediates key functions in the proangiogenic action of VEGF, although how VEGF induces the activation of FGD5 remains unknown. More importantly, the in vivo relevance of FGD5 to the pathogenesis of various angiogenesis-related diseases, such as atherosclerosis, diabetic retinopathy, and rheumatoid arthritis, is not known. Future studies are required to examine whether FGD5 could be a novel target for the treatment of those vascular diseases.

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Disclosures

None.

References

2. Garrett TA, Van Buel JD, Burridge K. VEGF-induced Rac1 activation in endothelial cells is regulated by the guanine nucleotide exchange factor Vav2. Exp Cell Res. 2007;313:3285–3297.
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Data Supplement

FGD5 Mediates Pro-angiogenic Action of Vascular Endothelial Growth Factor in Human Vascular Endothelial Cells

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Supplemental Figure 1. Regulation of the FGF-2-promoted cell proliferation by FGD5.
HUVECs, transfected with control or FGD5 siRNAs, were cultured in the presence or absence of FGF-2 or fetal bovine serum (FBS) and cell numbers were measured. The results shown are the means ± SE of six independent experiments. †, P<0.01; NS, not significant.
Supplemental Figure II. No effect of knockdown of FGD5 on VEGF-increased phosphorylation of Akt. HUVECs, transfected with control or FGD5 siRNAs, were cultured in the presence or absence of VEGF for the indicated minutes. Lysates of HUVECs were subjected to Western blotting using the indicated Abs. Representative images of three independent experiments are shown.

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