Molecular Cardiology

Endothelial Cell–Specific FGD5 Involvement in Vascular Pruning Defines Neovessel Fate in Mice

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Background—New vessel formation contributes to organ development during embryogenesis and tissue repair in response to mechanical damage, inflammation, and ischemia in adult organisms. Early angiogenesis includes formation of an excessive primitive network that needs to be reorganized into a secondary vascular network with higher hierarchical structure. Vascular pruning, the removal of aberrant neovessels by apoptosis, is a vital step in this process. Although multiple molecular pathways for early angiogenesis have been identified, little is known about the genetic regulators of secondary network development.

Methods and Results—Using a transcriptomics approach, we identified a new endothelial specific gene named FYVE, RhoGEF, and PH domain–containing 5 (FGD5) that plays a crucial role in vascular pruning. Loss- and gain-of-function studies demonstrate that FGD5 inhibits neovascularization, indicated by in vitro tube-formation, aortic-ring, and coated-bead assays and by in vivo coated-bead plug assays and studies in the murine retina model. FGD5 promotes apoptosis-induced vaso-obliteration via induction of the hey1-p53 pathway by direct binding and activation of cdc42. Indeed, FGD5 correlates with apoptosis in endothelial cells during vascular remodeling and was linked to rising p21^CIP1 levels in aging mice.

Conclusion—We have identified FGD5 as a novel genetic regulator of vascular pruning by activation of endothelial cell–targeted apoptosis. (Circulation. 2012;125:3142-3158.)

Key Words: angiogenesis-inducing agents ■ apoptosis ■ endothelium ■ FGD5 ■ models, animal

Vascularization during development and regeneration plays a vital role in adult disease progression, including tumor growth and metastasis, arthritis, diabetic retinopathy, and cardiovascular disease. Vascular growth in both development and disease consists of a strictly orchestrated, multi-step process that requires integrated activation of several molecular pathways. During early vascular growth, a dense primary vascular network without functional arterial and venous distinction is formed in response to low-oxygen conditions. This primitive system, consisting of small capillaries, is relatively unstable, with tip and stalk cell vessel structures expanding and collapsing at a high rate. Transition of this primary network into a stable secondary vasculature with a defined arterial/venous hierarchy of larger vessels that branch into a restricted capillary field requires intensive vascular remodeling, a late angiogenic process that includes neovessel stabilization and pruning of redundant vessel structures.1,2

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The molecular regulation by angiogenic factors such as vascular endothelial growth factor (VEGF)-A and fibroblast growth factor that promote growth of the primary vasculature has been studied extensively. In contrast, the key molecular pathways that regulate the reorganization of this early network into the more mature secondary vascular structure are still largely undefined. For the process of vascular pruning, vaso-obliteration by apoptosis induced by hyperoxia has been described,3 but little is known about the molecular regulation of this important aspect in vascular remodeling that determines the fate of the neovasculature. Here, we define the

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function of an endothelial cell (EC)–specific gene that plays a crucial role in apoptosis during vascular pruning.

Previously, we conducted a genome-wide expression profile analysis to identify potential trivial regulators in angiogenesis. A new gene named FYVE, RhoGEF, and PH domain–containing 5 (FGD5) was discovered that showed specific expression in endothelial precursor cells in developing murine embryos and vasculature of zebrafish larvae and mouse mice. The FGD family members of Rho guanine-nucleotide exchange factors (GEFs) include FGD1 through FGD6, as well as the FGD1-related cdc42-GEF,4–8 all sharing the DbI homology, FYVE, and pleckstrin homology (PH) domains. FGD1, FGD4, and FRG have been shown to control cdc42 activity via their DbI homology domain by converting inactive GDP-bound cdc42 into active GTP-bound cdc42, resulting in altered capacities in actin cytoskeleton assembly, filopodia formation, and Jun N-terminal kinase pathway activation.9–11 To date, the basic function of FGD5 remains unknown.

Our studies, which incorporate gain and loss of function of the FGD5 gene, demonstrated in vitro and in vivo that FGD5 induces vascular regression by pruning of redundant neovessels during neovascularization. FGD5 functions as a RhoGEF that binds and activates its direct target, cdc42, and therefore promotes Hey1-dependent p53-mediated apoptosis in ECs. The GFD5 mRNA level also correlates with rising p21^{CIP1} levels in aging C57/bl6 wild-type mice, which coincides with a decline in CD31 expression. These findings identify FGD5 as a novel, critical regulator of neovascular fate during late-phase angiogenesis and demonstrate that FGD5 may act as a defining factor for vascular regression in later adult life.

Methods
Isolation of Flk^+ and Flk^- Cells From Mouse Embryos
First, 10.5 to 15.5 days post coital FVB/N mouse embryos were harvested and digested to obtain single-cell suspensions in 0.12% collagenate type I/PBS in 10% FCS (Sigma C-0130; Sigma, Zwijndrecht, the Netherlands). Single-cell suspensions were labeled with 1:50 Flk1 phycophyrin mouse antibody and 1:100 Hoechst (BD Bioscience, Temse, Belgium) and harvested and digested to obtain single-cell suspensions in 0.12% collagenase type I (Sigma C-0130; Sigma, Zwijndrecht, the Netherlands). Single-cell suspensions were labeled with 1:50 Flk1 phycophyrin mouse antibody and 1:100 Hoechst (BD Bioscience, Temse, Belgium) and sorted to >95% purity for Flk^+ /H11546 and Flk^-/H11022 by flow cytometric assessment of the retinal ECs, which were double stained with Alexa Fluor–coupled isolectin IB4 (1:500; Invitrogen) and Alexa Fluor–labeled IgG antibody (Invitrogen, Breda, the Netherlands). Coverslips were double stained with Alexa Fluor–coupled isolectin IB4 (1:500; Invitrogen) and Alexa Fluor–labeled IgG antibody (Invitrogen, Breda, the Netherlands), and stained with 1:50 phycophyrin-labeled mouse anti-cdc42 (Abcam, Cambridge, UK). Cells were dissolved in RLT (RNA isolation) buffer and processed for quantitative polymerase chain reaction (qPCR) analysis.

qPCR and Western Blot Analysis
RNA was isolated with the RNAeasy kit (Qiagen, Venlo, the Netherlands), checked for quality and quantity by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Venlo, the Netherlands), and reversed transcribed into cDNA. qPCR reactions were performed by real-time fluorescence assessment of the SYBR Green signal with the iCycler IQ Detection System (Bio-Rad, Veenendaal, the Netherlands). qPCR analysis was performed for transcripts of mouse FRG, FGD1 through FGD6, endothelial nitric oxide synthase, p21^{CIP1}, and CD31, as well as for human VEGF receptor (VEGFR) 1/2, Notch1/4, DLL4, jagged1, ephrin B2/B4, NRPI/2, p53, and Hey1. Target mRNA expression levels are reported relative to the housekeeping genes, hypoxanthine guanine phosphoribosyl transferase (Hprt1) in murine samples and β-actin in the human samples, as previously described14 (All primer sequences are provided in Tables I and II in the online-only Data Supplement). For Western blot analysis, samples were lysed in NP40 buffer and analyzed on a 1.5% SDS-PAGE gel, followed by Western blotting with 1:1000 rat anti-p21^{CIP1} and anti-p53 antibody (Abcam, Cambridge, UK); 1:500 rabbit anti-cdc42, anti-Rac1, and anti-RhoA antibody (Abcam, Cambridge, UK); and 1:500 mouse anti-FGD5 antibody (Bioscience, Temse, Belgium) for protein detection. Protein bands were visualized with the Li-Cor detection system (Westburg, Leusden, the Netherlands) as previously described.15–17

Histological Analysis
Mouse ventricle, aorta, and carotid arteries were embedded in optical cutting temperature (Sakura Finetek, Hoge Rijndijk, the Netherlands) and snap-frozen in liquid nitrogen. Then, 5-μm cryosections were double stained with Alexa Fluor–coupled isocitron B14 (1:500; Invitrogen) and 1:100 mouse anti-FGD5 (Bioscience, Temse, Belgium), followed by FITC/rhodamine-labeled anti-mouse IgG antibody detection (1:500; Invitrogen, Breda, the Netherlands) and goat FAB against mouse IgG (H+L) for blocking (Jackson Immunoresearch Laboratories, Sacramento, CA) as previously described.18,19

Primary Cell Culture Conditions
Primary human umbilical vein ECs (HUCVECs; Lonza, Breda, the Netherlands) were cultured on gelatin-coated plates at 37°C/5% CO2 in EGM2 medium (EBM2 medium supplemented with commercial bullet kit and 2% FCS) with penicillin/streptomycin (Lonza, Breda, the Netherlands). Only cell cultures of passages 3 through 5 were used throughout the experiments. For immunohistological stainings, HUCVECs were grown on coverslips and fixed in ice-cold acetone for 5 minutes, followed by permeabilization in 0.1% Triton X/PBS, incubation with 1:100 mouse anti-FGD5 (Bioscience), and subsequent trichostatin A amplification of the signal (Roche, Woerden, the Netherlands). This was followed by incubation with 1:500 rabbit anti-cdc42 (Abcam, Cambridge, UK) or rat anti-zyxin (1:500; Abcam, Cambridge, UK) and detection with 1:500 FITC–labeled anti-rabbit/rat IgG antibody (Invitrogen, Breda, the Netherlands). Coverslips were mounted with Vectashield/DAPI (Brunschiwig, Amsterdam, the Netherlands) and imaged by fluorescence microscopy (Carl Zeiss Inc, Sliedrecht, the Netherlands).

Mouse Retinal Angiogenesis Model
Three-day-old C57bl/6 pups were anesthetized by placement on ice. Then, 0.5 μL adenovirus (Ad)-FGD5 (5×10^7 plaque-forming units [pfu]) was injected intravitreally into the left eye and 0.5 μL sham adenovirus (5×10^7 pfu) was injected into the right eye with a 33-gauge needle. For FGD5 silencing experiments, 100 nmol Accell siRNA targeting murine FGD5 was intravitreally injected into the left eye and 100 nmol Accell scrambled nontargeting siRNA was injected into the right eye (Dharmacon, Ettenleur, the Netherlands). For rescue experiments with VEGFR1, an active soluble form of VEGFR1 was co-injected with 100 nmol Accell siFGD5. Developing on the assay, pups were euthanized at post natal day 4, 6, 8, 11, 16, and 21, and retinas were stained with rhodamin/FITC isocitron IB4 (1:200) or rabbit anti–collagen IV antibody (1:200; Millipore, Amsterdam, the Netherlands), followed by Alexa Fluor–labeled anti-rabbit/mouse secondary antibodies (1:500; Invitrogen). Whole-mount retinas were visualized by confocal microscopy (LSM510 NLO/FCS; Zeiss, Silecpress, the Netherlands) using a 10× lens to obtain high-resolution micrographs of the topical vascular layer. Postprocessing was needed to reassemble the individual micrographs into an overview of the entire retina. Processed retina images were analyzed with a commercial analysis system (Angiosys, Bucking-ham, UK). At least 3 individual retinal flaps per mouse retina were assessed. Adequate transgene expression and gene silencing were validated by qPCR analysis. For flow cytometric assessment of the retinal ECs, the retina was homogenized in 0.12% collagenase type I (Sigma C-0130; Zwijndrecht, the Netherlands) in PBS/10% FCS for 15 minutes, filtered through a 3-μm mesh (BD Biosciences, Temse, Belgium), and stained with 1:50 phycophyrin-labeled mouse anti-
Flk1 antibody, followed by annexin V and propidium iodide (PI) staining (BD Biosciences, Temse, Belgium). The percentage of apoptotic cells in the Flk1⁺ population was quantified by flow cytometry (FACScanto; BD Biosciences, Temse, Belgium). For detection of cleaved caspase 3 and FGD5, retinal cells were fixed and permeabilized with the Cytofix/Cytoperm system (BD Biosciences, Temse, Belgium), followed by rabbit anti-FGD5 (Sigma, Zwijndrecht, the Netherlands) and secondary antibody staining with an allopheocyanin-labeled mouse anti-rabbit antibody, followed by FITC-labeled rabbit anti–cleaved caspase 3 and phycoerythrin-labeled mouse anti-Flk1 antibody staining and subsequent quantification by flow cytometry.

In Vivo Coated-Bead Assay
Mature severe combined immunodeficiency (SCID) mice (age, 10–15 weeks) were injected subcutaneously with 700 beads in 300 μL Matrigel (400 HUVECs per bead; BD Biosciences, Temse, Belgium) supplemented with 2.5 ng/mL rat fibrinogen and 20 ng/mL human basic fibroblast growth factor. For each animal, a sham virus–genic beads (Dynabeads, Invitrogen, Breda, the Netherlands) were coated and cross-linked with 2.5 μg anti-FGD5 antibody and an IgG-lisa detection system (Tebu-Bio, Heerhugowaard, the Netherlands). For validation of transgene expression, qPCR analyses were conducted on isolated samples by flow cytometry (FACScanto, BD Biosciences, Temse, Belgium).

Cell Proliferation, Apoptosis, and Cell Cycle Analysis
Transfected HUVECs were synchronized in the G0/G1 phase by serum deprivation in EGM2/0.2% FCS for 12 hours. For cell growth assessment, cells were harvested and quantified with a hemacytometer (trypan blue negative) at different time points. We performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide uptake experiments for cell metabolism assessment according to the manufacturer’s protocol (ATCC). For cell cycle analysis, cells were harvested at 0, 4, and 12 hours after activation; fixed in 70% ethanol/PBS for 15 minutes on ice; stained with PI (1:300); and analyzed by flow cytometry. For apoptosis analysis, cells were harvested at 0, 4, and 12 hours after activation and stained for annexin V and PI signals with an annexin V apoptosis detection kit (BD Biosciences, Temse, Belgium), followed by analysis of the samples by flow cytometry (FACScanto, BD Bioscience, Temse, Belgium).

Small G Protein Activation Assay and FGD5–Protein Complex Coimmunoprecipitation
GTP-RhoA, Rac1, and cdc42 activation levels were measured with the G-lisa detection system (Tebu-Bio, Heerhugowaard, the Netherlands). For FGD5–protein complex coimmunoprecipitation, magnetic beads (Dynabeads, Invitrogen, Breda, the Netherlands) were coated and cross-linked with 2.5 μg anti-FGD5 antibody (Biosciences) before immunoprecipitation overnight at 4°C with protein cell lysates of transfected HUVECs (50 μg total protein in 100 μL incubation buffer supplied by the G-lisa system). Beads were washed and protein samples were eluted with elution buffer (Invitrogen) before analysis by Western blot on a 12.5% SDS-PAGE gel.

Statistical Analysis
Results are expressed as mean±SEM, and statistical analysis was performed by 1-way ANOVA and Student t test. In specific assays, repeated measurement analysis was applied. A value of P<0.05 was considered significant.

Additional descriptions of the materials and methods used in this study are provided in the online-only Data Supplement.

Results
FGD5 Is Specifically Expressed in Endothelial Precursor Cells During Murine and Zebrafish Development and in Fully Differentiated ECs
FGD5 was specifically expressed in the Flk1⁺ cell population during embryonic development in mice as shown by microarray and qPCR. FGD5 mRNA was predominantly expressed in 8.5 days post coital until 16.5 days post coital Flk1⁺ endothelial precursor cells, when the majority of the vascular structures were established (Figure 1A). Selection of endothelial precursor cells based on the Flk1 cell surface marker was further validated by microarray analysis, which showed a significant rise in the expression levels of well-known angiogenic genes such as angiopoietin 2, neuropilin 1 and 2, Tie1, Flt1, and Ets1 in the Flk1⁺ compared with the Flk1⁻ pool (data not shown).

Specific vascular expression of FGD5 in the developing vascular tree was validated in zebrafish larvae by whole-mount in situ hybridization (Figure 1B). FGD5 mRNA in mature C57/bl6 mice was expressed predominantly in the aorta and carotid arteries compared with heart, skeletal muscle, kidney, liver, eye, and brain tissue (Figure 1C), mimicking the expression profile of endothelium-specific markers, including endothelial nitric oxide synthase and CD31. The other FGD family members showed a ubiquitous expression pattern (Figure 1D). In vitro, FGD5 was specifically expressed in primary human arterial ECs and HUVECs compared with nonrelevant cells (Hela and sarcoma cells; Figure 1E), underlining endothelium-specific expression throughout different species and developmental stages. Immunohistological analysis demonstrated selective FGD5 expression in ECs in the microvasculature of the myocardium and in the endothelial lining of large blood vessels, including the aorta (Figure 1F and 1G). These findings were further confirmed by analysis of public gene expression databases (NCBI, Gene Expression Omnibus; Figure 1 in the online-only Data Supplement). From these results, we conclude that FGD5 is the predominant member of the FGD family expressed in ECs during embryonic vascular development and in adult vasculature.

FGD5 Diminishes Angiogenesis In Vitro and In Vivo
FGD5 function in HUVECs was assessed by loss- and gain-of-function studies by transfection of FGD5-targeting siRNA or recombinant adenoviruses encoding for human or murine FGD5 cDNA, respectively (Figure II A–II E in the online-only Data Supplement). In a 2-dimensional Matrigel cord-formation assay, siRNA-mediated silencing of FGD5 promoted cord extension (Figure II F–III in the online-only Data Supplement), whereas FGD5 overexpression attenuated cord formation (Figure II I–II M in the online-only Data Supplement). Similarly, in an ex vivo murine aortic ring
assay, adenovirus-mediated overexpression of murine FGD5 significantly reduced vascular outgrowth (Figure IIN and IIO in the online-only Data Supplement). In an established angiogenesis model using HUVECs-coated Cytodex beads, FGD5 overexpression also diminished capillary sprouting and lumen formation (Figure IIP–IIU in the online-only Data Supplement). DAPI staining verified comparable numbers of cells attached to the beads at the initiation of the experiment (Figure IIV in the online-only Data Supplement).

To assess the role of FGD5 in angiogenesis in vivo, beads coated with Ad-FGD5 or sham adenovirus–transfected HU-VECs were suspended in Matrigel and injected subcutaneously in immunodeficient SCID mice. Matrigel FGD5 plugs harvested at day 8 after transplantation revealed reduced plug vascularization, whereas the sham plugs showed an extensive neocapillary network (Figure 2A–2D) and increased outgrowth of CD31+/H11001 cells (Figure 2E and 2F). Human FGD5 transgene expression in the plugs was validated at different time points by qPCR analysis and showed significantly higher levels of human FGD5 mRNA in plugs with Ad-FGD5– versus sham virus–treated HUVECs (Figure 2G). FGD5 function in angiogenesis was further assessed with an

![Assay](image1.png)

**Figure 1.** FGD5 is specifically expressed in endothelial cells. A, FGD5 expression in Flk1+/H11001 cells during embryonic development of C57/b6 mice from 8.5 to 16.5 days post coital analyzed by quantitative polymerase chain reaction (qPCR). FGD5 expression was upregulated in Flk1+/H11001 cells at all time points (n=6 for each time point). B, Whole-mount in situ hybridization of zebrafish larvae at 24 hours after fertilization revealed specific expression of FGD5 in the vasculature, including dorsal aorta, intersegmental vessels, and posterior cardinal vein. C, qPCR analysis of FGD5 expression in various tissues of mature C57/b6 mice (n=6 animals). Values represent mean±SEM. *P<0.05, aorta vs expression in all other tissues. D, qPCR analysis of the expression of the FGD family members FRG, FGD1, FGD2, FGD3, FGD4, and FGD6 and the expression pattern of vascular specific CD31 and endothelial nitric oxide synthase (eNOS; n=4 animals). Values represent mean±SEM. *P<0.05, aorta vs expression in various tissues. E, qPCR analysis of primary cell lines, including human umbilical vein and human arterial endothelial cells (HUVECs and HAECs), compared with nonrelevant cell types (Hela and sarcoma). Data obtained from 3 separate experiments. Values represent mean±SEM. The y axis represents arbitrary units (AU) showing target gene expression levels after correction with the housekeeping genes. *P<0.05, HUVECs and HAECs vs Hela and sarcoma cells. F, Immunohistological staining of the myocardium of mature C57/b6 mice demonstrated colocalization of the FGD5 protein (green FITC signal) with the EC marker isoelectin IB4 (red Cy-3 signal). Magnification ×4. G, Immunohistological staining of cryosections of aortas derived from mature C57/b6 mice. FGD5 is detected in the endothelium and adventitia (green FITC signal). No FITC signal was detected after coincubation with an isotypic control antibody. Magnification ×20. *Luminal area.
Figure 2. FGD5 inhibits angiogenesis in vivo: cell-bead plug assay. A, Representative macroscopic pictures of subcutaneously inoculated Matrigel plugs containing human umbilical vein endothelial cell (HUVEC)–coated Cytodex beads in severe combined immunodeficiency (SCID) mice at day 8. HUVECs were transfected with adenovirus (Ad)-FGD5, sham adenovirus, or nontransfected cells (control). B, Representative macroscopic pictures of the Matrigel plugs containing sham adenovirus and the Ad-FGD5–transfected EC beads in which the lack of vascularization and accumulation of erythrocytes within the plugs obtained from the Ad-FGD5 group are visible. C, Histological hematoxylin and eosin staining shows a decline in the accumulation of HUVECs on beads coated with Ad-FGD5–transfected HUVECs vs sham adenovirus–transfected HUVECs. White arrows point to perinuclear outgrowth. Magnification ¥4. D, Quantitative analysis of micrographs show the effect of FGD5 on the percentage of cell-covered plug area (mm² per bead) in the Ad-FGD5 group vs the sham adenovirus–transfected group and nontransfected controls (n = 8 animals per group; 20 beads per animal were analyzed). E, Immunohistological staining reveals accumulation of CD31/red Cy3⁺ ECs surrounding the Cytodex beads (left side), which colocalized with the blue fluorescent DAPI signal (right side). The contours of the Cytodex beads are visible by autofluorescence. Magnification ¥20. F, Quantitative analysis of the cell-bead plug data shows the effect of FGD5 on percent CD31⁺ surface area per bead (mm²) in the Ad-FGD5 group vs the sham adenovirus–transfected group and nontransfected controls (n = 8 animals per group; 20 beads per animal were analyzed). Values represent mean±SEM. *P<0.05 vs control and sham adenovirus groups. G, Validation of human FGD5
FGD5 Induces Apoptosis in ECs via an Hey1-p53 Regulatory Pathway

The observed antiangiogenic effects of FGD5 may be attributed to changes in EC proliferation and survival. FGD5-expressing HUVECs showed a reduced growth rate compared with sham virus–transfected controls (Figure IIIA and IIB in the online-only Data Supplement). In contrast, siRNA-mediated FGD5 knockdown in HUVECs increased cell proliferation compared with sisham controls (Figure IIC in the online-only Data Supplement). Annexin V/PI FACS analysis demonstrated that FGD5 overexpression promoted apoptosis, whereas siRNA-mediated FGD5 silencing diminished apoptosis (Figure IIIID and IIIE in the online-only Data Supplement). The increase in cell death coincided with rising p53 and p21CIP1 protein levels (Figure IIIF and IIIG in the online-only Data Supplement). These data imply that FGD5 could be involved in p53-dependent apoptosis and p21CIP1-associated cell cycle arrest, resulting in vascular regression. To elucidate the downstream signaling cascade involved in FGD5-mediated vascular pruning, the gene expression for well-defined angiogenic modulators was determined by qPCR. FGD5 knockdown inhibited VEGFR1/Flt1 and promoted VEGFR2/KDR/Flik1 mRNA expression, whereas FGD5 overexpression decreased VEGFR2 in favor of VEGFR1 mRNA levels, thus promoting an antiangiogenic state in the ECs (Figure IIIH in the online-only Data Supplement). In addition, FGD5 knockdown was associated with the downregulation of Notch pathway genes, including Notch1, Notch4, and DLL4. Hey1, one of the key downstream transcriptional regulators of the Notch signaling pathway,20 was markedly downregulated by FGD5 knockdown (Figure IIIH in the online-only Data Supplement). In contrast, FGD5 overexpression induced reversed effects. FGD5 knockdown or overexpression in HUVECs did not change mRNA expression of VEGF-A, Jagged1, NRP1, NRP2, ephrinB2, and ephB4 (Figure III in the online-only Data Supplement). To investigate whether the process of FGD5-mediated vascular pruning could be mediated by changes in the VEGFR1-to-VEGFR2 ratio, a soluble active form of VEGFR1 was provided in FGD5 siRNA–treated retinas. Indeed, the restoration of VEGFR1 bioavailability diminished the formation of excessive vascular structures that was associated with FGD5 silencing (Figure III in the online-only Data Supplement). Thus, FGD5 could regulate the vascular pruning process by promoting VEGFR1 versus VEGFR2 bioavailability.

Hey1 was previously associated with p53-mediated apoptosis,21 suggesting that Hey1 could mediate FGD5-induced apoptosis in ECs. Here, we assessed the function of Hey1 in FGD5-mediated apoptosis. Hey1 knockdown in FGD5-expressing HUVECs could rescue growth inhibition induced by FGD5 (Figure 5A). It also diminished p53 and p21CIP1 protein levels (Figure 5B and 5C) and reversed ECs from FGD5-induced apoptosis (Figure 5D and 5E). PI-aided cell cycle analysis suggested that FGD5 enhanced the sub-G1 apoptotic fraction, whereas the percent of alive and proliferating cells decreased (Figure 5F and 5G: ie, decrease in G1 and S+G2 fractions in the FGD5 group compared with the sham virus–treated group). This cytocstatic effect was rescued by Hey1 knockdown in FGD5-overexpressing ECs. Likewise, in an in vitro coated-bead assay, FGD5-mediated inhibition of capillary outgrowth was reversed by concomitant Hey1 silencing (Figure 5H and 5I).

Further studies demonstrate that FGD5-induced cell death was indeed mediated via p53 and p21CIP1 because cotransfection of Ad-FGD5 in HUVECs with p53- or p21CIP1-
Figure 3. FGD5 inhibits angiogenesis in vivo: murine retina model. A, Effect of murine FGD5 overexpression during retinal vascular development. Representative micrographs of the retinal vasculature visualized by whole-mount isoelectin IB4 staining from p8 to p21. Whole flat mount of the retina (left columns) and high-magnification examples of individual flaps (right columns) are displayed. Adenovirus (AD)-FGD5 injection at p3 severely impeded vascular development compared with sham adenovirus controls. Magnification ×10.

B

C

Nr. of Junctions

Mean Tube Length

Nr. of Tubes

Total Tube Length

Ad-FGD5

Sham Ad

P8

P11

P16

P21

Sham Ad

Ad-FGD5

Sham Ad

Ad-FGD5

Sham Ad

Ad-FGD5

Sham Ad

Ad-FGD5

Sham Ad

Ad-FGD5

Figure 3A. FGD5 inhibits angiogenesis in vivo: murine retina model. A, Effect of murine FGD5 overexpression during retinal vascular development. Representative micrographs of the retinal vasculature visualized by whole-mount isoelectin IB4 staining from p8 to p21. Whole flat mount of the retina (left columns) and high-magnification examples of individual flaps (right columns) are displayed. Adenovirus (AD)-FGD5 injection at p3 severely impeded vascular development compared with sham adenovirus controls. Magnification ×10.
Figure 3 (Continued). High-magnification micrographs show disruption of vessels and shortened sprouts. B, Representative examples of retinal flaps after processing by quantitative software (Angiosys); junctions (yellow dots) and vessels (blue lines) are visible. C, Quantification of this experiment showed that FGD5 overexpression induced a decrease in the number of tubes, number of junctions, and...
FGD5 Binds and Activates cdc42 Small GTPase

To determine the direct protein target of FGD5, we assessed whether FGD5 could bind and activate small GTPase proteins like its better-known family members. FGD5 transgene expression in HUVECs did not alter total cdc42, decreased Rac1, and increased RhoA protein levels (Figure 7A). However, communoprecipitation with an antibody against FGD5 identified cdc42 as the selective binding partner of FGD5 (Figure 7B), leading to specific activation of cdc42 in a GEF-activity assay (Figure 7C–7E). In addition, fluorescence microscopy showed colocalization of endogenous FGD5 with cdc42 at the perinuclear site in the cytoplasm of ECs (Figure 7F and 7G). In contrast, RhoA and Rac1 activity was significantly reduced in response to FGD5 overexpression. Because no direct binding of FGD5 to Rac1 or RhoA was observed in the communoprecipitation studies, these data suggest that the inhibition of Rac1 and RhoA activity is an indirect downstream effect of FGD5.

FGD5 Level Correlates With Vascular Regression in Aging

Transgenic mice with constitutively activated cdc42 show premature aging with failing DNA repair, accelerated cell senescence, and increased p53-dependent apoptosis.22 Similarly, cdc42 activation in cell culture was previously associated with cell senescence23 and apoptosis,24 whereas cdc42 deletion in hematopoietic stem cells promoted proliferation by loss of p21CIP1 regulation during cell cycle progression.25 To assess the role of FGD5 in aging-related vascular regres-
tion, we conducted qPCR analysis of aging wild-type C57bl/6 mice ranging from early fully mature (37–41 weeks) to old (80–92 weeks) specimens. A significant increase in GFD5 levels was observed in the oldest group of animals, which coincided with rising p21CIP1 expression, whereas CD31 levels were diminished in the oldest group (see Figure IV in the online-only Data Supplement).

Discussion

This study has several findings. First, FGD5 is expressed specifically in both progenitor and mature ECs, in contrast to its family members FRG, FGD1, FGD2, FGD3, FGD4, and FGD6, which show a ubiquitous expression in different cell types. In addition, this EC-specific expression is preserved throughout the evolution in different species. Second, FGD5 inhibits neovascularization, as indicated by the results obtained in vitro from tube-formation, aortic-ring, and coated-bead assays. These findings were validated in vivo by results obtained from the coated-bead plug assay and the murine retina model. Third, FGD5 binds and activates its direct downstream target, cdc42. Fourth, FGD5 inhibits neovascularization by apoptosis-induced vaso-obliteration via induction of the hey1-p53 pathway. Finally, FGD5 indeed correlates with apoptotic marker expression in Flk1+ ECs during vascular remodeling in the mouse retina, whereas high levels of FGD5 expression were linked to rising p21CIP1 mRNA levels in aging C57bl6 wild-type mice.

Here, we define, for the first time to the best of our knowledge, the function of FGD5 as an EC-specific GEF that plays a crucial role in apoptosis-induced vascular pruning during vascular remodeling.

Vascular growth and remodeling during development and disease are tightly regulated by stimulatory and inhibitory signals that determine the complexity of the vascular tree hierarchy. Here, we describe the inhibitory role of FGD5 in vascular development, a member of the FGD family of RhoGEFs from which thus far no biological function was allocated. FGD1 was the first FGD member to be identified, and mutations in the gene were proven to be responsible for faciogenital dysplasia or Aarskog-Scott syndrome.8 Further studies indicated that FGD1 functioned as a cdc42-specific GEF, thereby activating cdc42 signaling by exchanging bound GDP with GTP.7 FGD2, FGD3, FGD4, and FRG were identified by genetic searches as FGD1 homologs4–6 and were all subsequently associated with a specific role in cdc42 activation with downstream effects on cell migration and morphology,9–13 whereas the function of FGD5 and FGD6 remained to be elucidated. Although the preserved cdc42 GEF activity of the different FGD members seemed to imply a redundancy in the protein family by overlapping function, the specific expression of FGD5 in ECs points toward a unique role for this particular FGD member in EC regulation. Similar to the other family members, FGD5 comprises (in order) a Dbl homology adjacent to a PH domain, followed by a FYVE-finger domain and a second C-terminal PH domain.6,12,13 Here, we show specific direct binding of FGD5 to cdc42 and
Figure 5. FGD5 induces apoptosis in endothelial cells via Hey1 activation. A, Number of human umbilical vein endothelial cells (HUVECs) at 3 days in sham virus–, adenovirus (Ad)-FGD5–, Ad-FGD5 / Hey1 siRNA, Ad-FGD5 / sham siRNA–, and sham virus / sham.
demonstrate FGD5-mediated activation of cdc42. In addition, similar to recent findings that indicated colocalization of FGD1 with cdc42 in the Golgi complex in Hela cells, FGD5 intracellular distribution in ECs was colocalized primarily with cdc42 in the perinuclear region. Together, these data identify cdc42 as the direct downstream target of FGD5 and assign an EC-specific RhoGEF function to this gene.

Our data also demonstrated that the effect of FGD5 could be attributed to p21cip1-mediated G1/G2 cell cycle arrest, followed by p53-mediated apoptosis. Transgenic overexpression of FGD5 coincided with the upregulation of the Notch signaling pathway, including Notch1, Notch4, DLL4, and the downstream transcription factor hey1. Genetic regulation of these Notch genes could be induced after FGD5-mediated
Figure 6. FGD5-mediated cell death depends on p21CIP1 and p53 signaling. A, Dot plot graphs show apoptosis in human umbilical vein endothelial cells (HUVECs) transfected with sham virus, adeno virus (Ad)-FGD5, and Ad-FGD5 with siRNA targeting p53 or p21CIP1. B, Quantification of the percentage of annexin V+ cells. Values represent mean±SEM *P<0.05 vs sham virus, Ad-FGD5 with siRNA p53, and Ad-FGD5 with siRNA p21CIP1 (data obtained from 4 different experiments). C, Analysis of cell cycle distribution of nontransfected...
cdc42 activation via the p38 mitogen-activated protein kinase signaling pathway that regulates transcriptional activation. More downstream of cdc42 toward the process of apoptosis, recent studies have indicated that hey1 triggered p53 activation through repression of HDMD2 transcription, identifying a novel direct link between hey1 and p53. Our experiments further showed that siRNA targeting of hey1 in FGD5 transgenic ECs inhibited FGD5-induced G1/G2 cell cycle arrest and cell death by reverting protein levels of p53 and p21CIP1 back to baseline. In addition, hey1 knockdown of FGD5 transgenic HUVECs restored the sprouting capacity of these cells in the in vitro coated-bead assay, whereas siRNA targeting of p53 or p21CIP1 in FGD5 transgenic ECs obtained similar effects on apoptosis and sprout formation. Together, our data clearly indicate FGD5-mediated cell death in ECs involves the hey1-p53-p21CIP1 pathway.

In our studies, we demonstrated that rising endogenous FGD5 levels in the Flk1+/H11001 ECs in the developing retinal vasculature were associated with the expression of the apoptosis marker cleaved caspase 3. Surprisingly, a large population of Flk1+/casp3+/FGD5− cells was also observed next to the Flk1+/casp3+/FGD5+ subpopulation, which suggests that FGD5-induced apoptosis is not the only regulatory mechanism of vascular pruning. Indeed, Ishida et al have previously published that vascular regression in the murine retina was also regulated by infiltration of leukocytes. These cells induce apoptosis of the redundant vascular structure via a Fas ligand–mediated process. In light of these findings, our data demonstrate that FGD5 activation provides an additional mechanism that is partially responsible for the observed cell death of Flk1+/ECs in the natural vascular pruning process.

Wang and coworkers demonstrated that during natural aging, cdc42 activity in different organs was increased in wild-type mice. By targeting cdc42GAP (which reverses cdc42 from its active GTP-bound to its inactive GDP-bound state), they successfully created a murine strain with constitutively elevated cdc42-GTP levels. These cdc42GAP-null animals had a prematurely aged phenotype with a failing DNA repair system and a considerably shortened average lifespan from 27 to 12 months. In addition, increased apoptosis was observed in various cell types during organ development and aging, which was dependent on p53 and p21CIP1 activation. Cdc42GAP-null cells also showed early proliferative senescence compared with wild-type cells. Cdc42 activation was previously associated with cell senescence and apoptosis, whereas cdc42 deletion in hematopoietic cells...
Figure 7. FGD5 binds and activates cdc42 small GTPase. A, Western blot analysis of protein levels of cdc42, rac1, and RhoA after FGD5 expression in human umbilical vein endothelial cells (HUVECs). B, Coimmunoprecipitation (Co IPP) of FGD5 in cell lysates derived from FGD5 overexpressing HUVECs. Western blot analysis of the samples showed selective coprecipitation of cdc42, but not Rac1 or RhoA, whereas coimmunoprecipitation with a control IgG isotype showed no effective coprecipitation. Shown are representative Western blots from 3 different experiments. Chemiluminescence measurement of the GTP-bound small G proteins in cell lysates from sham adenovirus- or Ad-FGD5–transfected HUVECs showing the levels of (C) GTP-Rho-A, (D) GTP-Rac1, and (E) GTP-cdc42 in response to serum activation (data obtained from 4 separate experiments in duplicate). Values represent mean ± SEM. *P < 0.05 vs sham virus as assessed by repeated measures analysis. F, Representative micrographs of 3 different experiments showing perinuclear localization of FGD5 (red fluorescent signal; nucleus stained by blue DAPI) in the cytoplasm of HUVECs. FGD5 signal colocalizes with cdc42 (green fluorescent signal) at the perinuclear site. Magnification ×40. G, FGD5 (red signal) does not colocalize with the focal adhesion marker zyxin (green signal). Magnification ×40.
FGD5 silencing promoted survival of excessive vascular pruning induced EC apoptosis and vascular regression, whereas FGD5 silencing promoted survival of excessive vascular structures.

Thus far, we have established an important role for FGD5 in vascular remodeling as an inducer of vascular pruning of redundant neovessels. In aging, an imbalance between cdc42 deactivation and activation could result in elevated cdc42 activity, which has severe effects on the proliferative and stress-responsive capacities of aging cells. Thus, it would be interesting to study the level of FGD5 expression not only in diseases with pronounced apoptosis-induced vascular regression but also in the aging vasculature. In this study, we found a correlation between FGD5 levels and increasing levels of p21(cip1) expression in the highly vascularized hind-limb tissue of aging wild-type mice, which coincided with a decline in CD31 EC marker expression. Currently, we are conducting further studies to elucidate the role of this EC-specific guanine nucleotide exchange factor in vascular regression in the natural aging process.

Conclusions

We have identified FGD5 as a novel genetic regulator of vascular pruning of redundant neovessels by activation of targeted apoptosis. In addition, FGD5 function could prove to be a decisive factor in the survival and stability of aging vasculature.

Acknowledgments

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Disclosures

None.

References

1. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development. 1998; 125:1591–1598.


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**CLINICAL PERSPECTIVE**

The capacity of endothelial cells in the vasculature to regenerate in human adults depends largely on the same molecular pathways that regulate the process of angiogenesis during embryonic development. Growth of neovessel not only is determined by the availability of growth factors such as vascular endothelial growth factor-A but also depends on the ability of the neovascular structure to maintain stability while excess vessel structures are actively removed by a biological mechanism called vascular pruning. In this article, a novel factor named FYVE, RhoGEF, and PH domain–containing 5 (FGD5) was discovered to be involved in active regulation of this pruning process. A better understanding of the molecular pathways that govern vascular pruning could benefit patient treatment in therapies in which stimulation of vascular growth is desired and in which this type of regulation should be adapted.
Endothelial Cell–Specific FGD5 Involvement in Vascular Pruning Defines Neovessel Fate in Mice


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Correction

In the article by Cheng et al, “Endothelial Cell-Specific FGD5 Involvement in Vascular Pruning Defines Neovessel Fate in Mice,” which was published in the June 26, 2012 issue of the journal (Circulation. 2012;125:3142–3159), Figure 3B was incorrect.

The correct Figure 3B is:

![Figure 3B](image_url)

The figure has been corrected in the current online version of the article.

The authors regret the error.
Supplemental Material

Figure legends

Figure 1. Amongst the FGD-family members, FGD5 is uniquely expressed in progenitor and mature endothelial cells. (a) Microarray analysis of Flk1+ cells as compared to Flk1- cells isolated from murine embryoid bodies at different time points. FGD5 expression levels are shown in the bar-graphs. (b-f) Microarray analysis of different types of cultured human cells (ECs including microvascular EC (MVEC), arterial EC (AEC), and venous EC (VEC)), epithelial cells (E), fibroblasts (F), smooth muscle (SM), and stromal cells (SC) derived from different organs. Bar-graphs indicate the expression levels of (b) FGD5, (c) FRG, (d) FGD1, (e) FGD3, and (f) FGD6. Shown are values of the mean ± SEM.

Figure 2. siRNA and adenoviral transfection efficiency. (a) In vitro transfection of HUVECs with siGlo demonstrated a transfection efficiency of ~90% for siRNA identified by an intracellular green fluorescent signal. (b) Adenovirus-mediated transfection of Ad-LacZ gene showed a transfection efficiency of ~95% at moi 100 in HUVECs as shown by β-gal staining. (c) qPCR analysis for validation of FGD5 silencing by FGD5-targeting siRNA showed significant FGD5 downregulation compared to non-targeting scrambled siRNA (shamsi) transfected (~80% decrease), or non-transfected HUVECs (~60% reduction). (d) QPCR analysis revealed that transfection of an adenoviral vector expressing human FGD5 cDNA at moi 100 significantly increases FGD5 expression compared to non-transfected and sham virus (sham Ad) transfected controls. (e) Western blot analysis showed upregulation of FGD5 at protein level
following Ad-FGD5 transfection. **FGD5 inhibits angiogenesis in vitro and ex vivo.** (f) Representative tube formation of HUVECs in a standard 2D Matrigel cord formation assay following FGD5 silencing or sham siRNA transfection. HUVECs were visualized by Calcein-AM uptake. 4X magnification. Quantitative analysis of the Matrigel assays show the effect of FGD5-knockdown (FGD5KD) on (g) total tube/cord length, (h) number of cords/tubes, and (i) number of junctions compared to control and sham-treated HUVECs (N=6 individual experiments). Values represent means ± SEM. *P<0,05 FGD5KD versus control and sham siRNA, #P<0,10 FGD5KD versus control and sham siRNA. (j) Representative cord formation of HUVECs in a standard matrigel assay following FGD5 overexpression using Ad-FGD5, or sham adenovirus (Sham Ad). HUVECs were visualized using Calcein-AM uptake. 4X magnification. Quantitative analysis of the Matrigel assays demonstrate the effect of FGD5 overexpression on (k) total tube/cord length, (l) number of tubes/cords, and (m) number of junctions compared to control and sham adenovirus-transfected HUVECs (N=6 individual experiments). Values represent means ± SEM. *P<0,05 Ad-FGD5 versus control and sham adenovirus. #P<0,1 Ad-FGD5 versus control and sham adenovirus. (n) Representative micrographs show microvascular sprouting of matrigel embedded aortic rings transfected with Ad-FGD5 or sham adenovirus. 4X magnification. (o) Quantitative analysis of the aortic ring assays demonstrate the effect of FGD5 overexpression on the microvascular surface area (N=8 individual aortic explants). Values represent means ± SEM. *P<0,05 Ad-FGD5 versus sham adenovirus. (p) FGD5 expression impedes microvascular sprouting in a coated-bead assay. Representative micrographs of microvascular sprouting of HUVECs coated on cytodex beads in matrigel. HUVECs were transfected with Ad-FGD5 and compared to non-transfected and sham adenovirus-treated controls. 20X magnification.
Representative micrographs of phalloidin-stained microvascular networks (Texas-red fluorescent signal) of the non-transfected, sham virus transfected, and Ad-FGD5 transfected groups. 20X magnification. Quantitative analysis of the micrographs demonstrates the effect of FGD5 overexpression on the relative sprout area per bead, and the number of beads with multiple sprouts. Toluidine blue staining shows the effect of FGD5 on lumen formation in the formed vessels. 20X magnification. Quantitative analysis of the micrographs shows the effect of Ad-FGD5 overexpression on the number of beads with lumen formation compared to non-transfected and sham adenovirus-transfected controls (N=6 separate experiments, analysis of 20 beads per group per experiment). Values represent means ± SEM. *P<0.05 versus control and sham adenovirus. †P<0.05 versus control. Comparable coating efficiency of Sham Ad and Ad-FGD5 transfected HUVECs on cytodex beads. Nuclei were visualized by DAPI staining. High resolution pictures of representative whole mount retinas stained with Isolectin IB4-FITC at p8, comparing the effect of FGD5 overexpression induced by injection of AdFGD5 at p3, to the effect of FGD5 silencing induced by injection of siFGD5 at p3, assessed in comparison to their appropriate controls (sham Ad and sisham).

**Figure 3. FGD5 inhibits EC proliferation and promotes programmed cell death**

Cell growth in HUVECs transfected with Ad-FGD5 (black triangle) compared to sham adenovirus-transfected (white square), or non-transfected controls (black circle). The number of cells was quantified every 24 hours. The graph shows the effect of FGD5 on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) processing in HUVECs. Cell count of HUVECs treated with siRNA targeting FGD5
compared to control siRNA treated or non-treated controls, at 3 days post transfection (Data was obtained from 4 separate experiments in triplicate). Values represent means ± SEM. *P<0.05 versus sham adenovirus/sham siRNA and non-treated control. (d) Flow cytometry evaluation of apoptosis in HUVECs transfected with Ad-FGD5 versus sham virus treated cells (upper panel), and flow cytometry evaluation of apoptosis in non-transfected HUVECs (control), sisham and siFGD5 transfected cells (lower panel). (e) Quantification of total percentage of Annexin V+ cells of adFGD5 versus sham virus treated cells (left graph), and siFGD5 versus sisham treated cells (right graph). (Data obtained from 4 different experiments). *P<0.05 versus sham virus-transfected cells and versus control and sisham-transfected cells respectively. Representative western blot analysis of (f) p53 and (g) p21CIP1 levels in Ad-FGD5 and sham adenovirus-transfected HUVECs 2 days post transfection. Graphs show Licor-quantified differences in band density (Data obtained from 3 separate experiments. Values represent means ± SEM). *P<0.05 versus sham adenovirus-transfected cells. (h) qPCR analysis of RNA expression of VEGFR1, VEGFR2, and Notch signalling pathway genes following FGD5 overexpression and silencing compared to the appropriate controls. (i) Expression levels of NRP1, NRP2, ephB4, EphrinB2, Jagged1, and VEGFA were assessed in Ad-FGD5 versus Sham Ad transfected cells, and in FGD5-knockdown versus sisham. Data was obtained from 6 separate experiments in duplicates. Shown are values of the mean ± SEM. (j) FGD5 mediated pruning is partially regulated by VEGFR1. Effect of co-treatment with an active soluble form of VEGFR1 during siRNA mediated silencing of endogenous FGD5 in murine retinal vascular development. Representative micrographs of the retinal vasculature visualized by whole mount isolectin IB4 staining at p8. Individual flaps are displayed. Injection of VEGFR1(sol) at p3 reversed the effect of
siFGD5. Excessive vascular structures that were associated with FGD5 silencing were significantly reduced as compared to the vascular network of retinas injected with siFGD5 only. 10X magnification. Quantification of this experiment showed that treatment with VEGFR1(sol) during FGD5 silencing decreased the number of junctions and tubes, and total tube length (µm), whereas an increase was observed in mean tube length (µm) (N=8 pups per condition). Values represent means ± SEM. *P<0.05 versus siFGD5-injected eye.

**Figure 4. FGD5 expression levels correlate with increased p21\textsuperscript{CIP1} expression in aging**  
Tissue samples of the hindlimb muscles of C57/bl6 mice of 37-41 wks, 65-66 wks, and 90-92 wks of age were collected and processed for qPCR analysis. Shown here are the expression levels of (A) FGD5, (B) p21\textsuperscript{CIP1}, and (C) CD31 in the different age groups. Data was obtained from at least 8 animals per group, qPCR was performed in duplicate. Shown are values of the mean ± SEM. *P<0.05 versus 37-41 wks, **P<0.05 versus 65-66 wks.
Material and Methods

**Adenoviral vector mediated gene transfer and siRNA-knockdown in cell culture**

The commercial pAd/CMV/V5 Gateway system (Invitrogen, The Netherlands) was used to generate the recombinant adenoviral vectors according to the manufacturer’s recommendations and was expressed in 293 cells. Viral particles were harvested from cell lysates and the viral titer was determined by viral plaque assays. ΔE1/E3-sham adenovirus was used as a control adenovirus (sham Ad) in all studies. 60% confluent HUVEC-cultures were infected in EGM2/0.2% FCS for 2 hours at 37°C/5% CO₂ (moi 100), resulting in >90% transfection efficiency at 48 hours. Targeted knockdown of genes (Hey1, p53, FGD5 and p21CIP1) was achieved by transfer of a mix of 4 specific siRNAs sequences directed against the target mRNA (Smartpool, Dharnacon, The Netherlands) in 50-60% sub-confluent HUVEC cultures, at 3 days prior to inclusion in experiments. As a control, cells were transfected with a mix of 4 scrambled non-targeting siRNAs (Dharnacon, The Netherlands). siRNA transfection efficiency of >80% of HUVECs was achieved at 72 hours, as validated by FITC-labelled siRNA (siglow, Dharnacon, The Netherlands). Adequate overexpression or knockdown of the target genes was validated by qPCR and western blot analysis at 2 and 3 days post transfection respectively.

**In vitro and ex vivo angiogenesis assays**

**Tube-formation assay**: HUVECs were seeded at a density of $3 \times 10^4$ cells/ml in 200 μl EGM2 medium in a 96-well plate on serum-reduced Matrigel (BD Biosciences, The Netherlands) and incubated for 24 hours. Viable cells were visualized by Calcein-AM
uptake according to the manufacturer’s protocol (BD Biosciences, The Netherlands) and fluorescence microscopy. **Coated-bead assay:** Trypsinized single cell suspensions of HUVECs (24 hours post-transfection) were co-incubated for 4 hours with Cytodex microcarrier beads (Sigma, The Netherlands) at a ratio of 400 cells per bead in EGM2 medium, as described previously. HUVECs-coated micro-beads were transferred to a 6-well plate and incubated overnight in EGM2 medium, followed by 2 washes to eliminate unattached cells. Coated micro-beads were resuspended in 2.5% human fibrinogen/ml EGM2 (Calbiochem, The Netherlands) supplemented with 1 µg thrombin (R&D Systems, UK), and plated in 12-well plate. Coagulated fibrinogen gels were covered by 1 ml EGM2 medium supplemented with 20ng/ml bFGF, before visualization by rhodamin-phalloidin staining (Invitrogen, The Netherlands) or tuloidine blue staining at day 6 of culture. **Ex vivo analysis of endothelial sprouting:** Aortas of C57bl/6 mice were harvested, cut into 2 mm thick segments, and placed in serum-reduced Matrigel in a 96-well plate (BD Biosciences, The Netherlands). Imbedded aortic segments were then incubated with 200 µl EGM2 medium per well for 4 days before evaluation by phase-contrast microscopy. **Quantification of the tube-formation assay:** Angiosys analysis software (Angiosystems, UK) was used to determine mean and total tube length, and the number of tubes and junctions per field of view. **Quantification of the coated-bead and aortic ring assay:** Data analysis was performed using a commercial image analysis system (Impak C, Clemex Technologies, Canada).
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Figure 4 part 1 of 1