MATERIALS AND METHODS

Cell culture and transfection

Primary HUVECs were isolated from umbilical cords obtained through local hospitals under University of California, Irvine Institutional Review Board approval. HUVECs were routinely cultured in M199 (Gibco) supplemented with 10% fetal bovine serum (FBS) and endothelial cell growth supplement (BD Biosciences) at 37°C and 5% CO_2. Normal human lung fibroblasts (NHLFs; Lonza) were routinely grown in M199 supplemented with 10% FBS at 37°C and 5% CO_2. HUVECs between P3 and P4 and NHLFs between P6 and P10 were used for all experiments. ECs at 80% confluency were transfected with 40 nM siRNA (Invitrogen) for 2 hours or 13 nM siRNA (Ambion) or 4 µg DNA for 4 hours using Lipofectamine 2000 in Opti-MEM (Invitrogen). Following the incubation, the transfection mixture was replaced with fresh growth medium and cells were allowed to recover overnight. For angiogenesis assays, transfected ECs were allowed to recover overnight in EGM-2 medium (Clonetics). Cells were used for experiments 18 to 24 hours after transfection. Stealth RNAi siRNAs to human IFITM1 and IFITM3 were purchased from Invitrogen and the sequences are as follows: IFITM1, 5'-GGCUCUGUGACAGUCUACCAUAUUA-3' (sense) and 5'-UAAUAUGGUAGACUGUCACAGAGCC-3' (antisense); IFITM3, 5'-UGAAUCACACUGUCCAAACCUUCUU-3' (sense) and 5'-AAGAAGGUUUGGACAGUGUGAUUCA-3' (antisense). Silencer select siRNAs to human IFITM1 were purchased from Ambion and the sequences are as follows: IFITM1 #2, 5'-GCCCAUAGCCUGCAACCUUtt-3' (sense) and 5'-AAGGUUGCAGGCUAUGGGCgg-3' (antisense). Non-targeting stealth RNAi siRNA negative control medium GC duplex (Invitrogen) or silencer select negative control #1 siRNA (Ambion) were used as respective controls in all experiments.

Retroviral vector construction and EC transduction

The open reading frame of human IFITM1 with an N-terminal FLAG-tag was inserted into the multiple cloning site of pBMN-GFP retroviral vector (Orbigen) using standard cloning techniques. Retroviral transduction of ECs was performed using the Phoenix retrovirus expression system (Orbigen), per manufacturer's instructions, with some modifications. First, retroviral producer cells (293T, Phoenix Ampho; Orbigen) were transfected with 4 µg of pBMN-GFP or pBMN-GFP-FLAG-IFITM1 retroviral vectors for 6 hours using Lipofectamine 2000 in Opti-MEM and allowed to recover in DMEM (Gibco) containing 10% FBS overnight at 32°C and 5% CO_2. Transfection efficiencies (GFP-positive cells) were consistently >90%. Viral supernatants were harvested 48 hours later, passed through a 0.45 µm filter, and 5 mL was added, together with 8 µg/mL polybrene (Invitrogen), to low-density ECs in 10 cm culture dishes. After 6 hours, viral medium was replaced with M199 supplemented with 20% FBS and endothelial cell growth supplement. This procedure was repeated daily for three consecutive days. Transduction efficiencies (GFP-positive cells) were consistently between 70-90%.

Real-time quantitative RT-PCR

Total RNA was isolated from ECs using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Following treatment with RQ1 DNase (Promega), 1 µg of total RNA was synthesized to cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative reverse-transcription PCR (qRT-PCR) was performed on iCycler iQ5 (Bio-Rad) using SYBR Green (Molecular Probes) and HotStarTaq DNA Polymerase (Qiagen). Cycling parameters available upon request. All samples were measured in triplicate and average C_T values normalized to GAPDH expression levels. Standard curves were used to determine relative copy number. All data analyses were performed using iQ5 software (Bio-Rad). qRT-PCR primers were ordered from Integrated DNA Technologies and the sequences are as follows: IFITM1, 5'-

CCAGCATCCGGACACCACAG-3' (upper) and 5'-CCCCCAGCACAGCCACCTC-3' (lower); IFITM2, 5'-CGTCCAGGCCCAGCGATAGAT-3' (upper) and 5'-GCAGGGCGAGGAATGGAAGATAG-3' (lower); IFITM3, 5'- CGCCCCACAACCCTGCTC-3' (upper) and 5'-ACGTCGCCAACCATCTTCCTG-3' (lower); GAPDH, 5'-TCGACAGTCAGCCGCATCTTCTT-3' (upper) and 5'-GCGCCCAATACGACCAAATCC-3' (lower).

Angiogenesis assays

In vitro angiogenesis assays were performed as described.[1] Briefly, ECs were coated onto Cytodex 3 microcarrier beads (Amersham) at a concentration of 100 cells/bead for 4 hours and then incubated overnight in EGM-2 medium (Clonetics). The following day, EC-coated beads were washed briefly in EGM-2 and suspended in a 2.5 mg/mL fibrinogen solution (MP Biomedicals) at a concentration of 500 beads/mL. 0.5 mL of the fibrinogen/bead suspension was then added to each well of a 24-well plate containing 0.5 U of thrombin (Sigma-Aldrich). After the gels clotted, 1 mL of EGM-2 containing 20,000 NHLFs was added to each well and cultures were maintained for desired number of days. On day 6 of the assay, live cultures were observed under bright field for quantification. The number of sprouts per bead and the percentage of lumenized sprouts per bead were quantified and averaged for 30 beads per condition. A sprout was defined as a vessel greater than or equal to the diameter of the bead in length. A lumenized sprout was defined as a vessel containing a continuous lumenal space along the entire length of the vessel. To isolate ECs from angiogenesis assays for qRT-PCR, microarray, and western blot, NHLF monolayers were removed from the gels with 5 mg/mL trypsin (Sigma-Aldrich) and then gels were digested with 10 mg/mL trypsin to release the EC-coated beads. The entire contents of 24 wells were combined, centrifuged at 1,200 rpm, and the resulting pellet containing ECs and beads was suspended in the appropriate buffer.

Western blot

ECs were isolated from angiogenesis assays as described above and suspended in 500 µL cold lysis buffer (20 mM Tris-HCl, pH 7.9, 137 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1X protease inhibitor cocktail (Roche)) or lysed directly in culture dishes. Cells were lysed on ice for 10 minutes, sonicated for 10 seconds at 10 watts, and cellular debris were removed by centrifuging at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined using bicinchoninic acid assay (Sigma-Aldrich) according to manufacturer's instructions. Samples were mixed 1:1 with Laemmli sample buffer (Bio-Rad), boiled for 5 min at 95°C, and equal amounts of protein (40-60 µg) were loaded and electrophoresed in 4-20% Mini-PROTEAN TGX polyacryalamide gels (BioRad) under denaturing and reducing conditions. Proteins were transferred to polyvinylidene fluoride membranes (Millipore) and membranes were blocked in TBS/0.1% Tween 20 (TBST) containing 3% milk for IFITM1 and FLAG, 5% BSA for occludin, or 1% BSA/3% milk for claudin-5 for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with rabbit polyclonal anti-IFITM1 antibody (PA000192-PA1112; Syd Labs) diluted 1:500 in 3% BSA/TBST, mouse monoclonal anti-FLAG antibody (F1804; Sigma-Aldrich) diluted 1:1000 in 3% milk/TBST, mouse monoclonal anti-occludin antibody (33-1500; Novex) diluted 1:1000 in 5% BSA/TBST, or rabbit polyclonal anti-claudin-5 (ab53765; Abcam) diluted 1:2000 in 1% BSA/3% Milk. The following day, membranes were incubated with an HRP-conjugated goat anti-rabbit (ab6721; Abcam) or anti-mouse (sc-2060; Santa Cruz) secondary antibody diluted 1:5000 in 3% BSA/TBST for 2 hours at room temperature. To check for equal loading, membranes were probed with an HRP-conjugated β-actin antibody (ab20272; Abcam). Blots were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and imported into NIH ImageJ for densitometry quantification.

Coimmunoprecipitation

The open reading frame of human IFITM1 with an N-terminal FLAG-tag was inserted into the multiple cloning site of pcDNA3.1(+) vector (Invitrogen) using standard cloning techniques. ECs were transfected with vector or FLAG-IFITM1 as described above and grown to 100% confluency. Cells were lysed in cold 1% CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1X protease inhibitor cocktail). Lysates were rotated at 4°C for 30 minutes and centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatants were collected and pre-cleared using protein A/G beads (Santa Cruz). Pre-cleared lysates were incubated with mouse monoclonal anti-FLAG or mouse IgG (sc-3877; Santa Cruz) antibodies overnight at 4°C by end-over-end rotation. The next day, protein A/G beads were added and the samples were rotated end-over-end for 3 hours at 4°C. Lysates were centrifuged at 4,000 rpm for 5 minutes at 4°C and supernatants discarded. Beads were washed 4 times with cold 1% CHAPS lysis buffer. Samples were analyzed by western blot as described above.

Lumenogenesis assays

In vitro lumenogenesis assays were performed as described.[2] Briefly, ECs suspended in rat-tail collagen type I matrices (3.75 mg/mL) at a concentration of 6 x 10^5 cells/mL were added at 30 µL per well to 4.5 mm diameter microwells (Corning) and incubated for 30 minutes at 37°C to polymerize the gels. Serum-free culture medium of M199 containing 1X ITS+3 (Sigma-Aldrich), 40 ng/mL VEGF (293-VE; R&D Systems), 40 ng/mL FGF-2 (233-FB; R&D Systems), 50 µg/mL ascorbic acid (Fisher Scientific), and 50 ng/mL PMA (Calbiochem) in a volume of 100 µL was added to each well. For quantification of EC lumens, cultures were fixed in 3% glutaraldehyde for 30 minutes and stained with 1% toluidine blue in 30% methanol for 1 hour. Four bright field images (three microwells per condition) were analyzed using NIH ImageJ software. An EC lumen was defined to include both multicellular lumens and intracellular vacuoles. The area of each lumen was manually traced, converted from pixels to µm^2, and averaged for each condition.

Fluorescent labeling of intracellular vacuoles

Lumenogenesis assays were performed as described above, but 5 mg/mL FITC-conjugated dextran (10,000 MW, anionic; Molecular Probes) was added to the culture medium. After 4 hours, gels were digested with 5 mg/mL collagenase Type I for 10 minutes at 37°C. Cells from 3 microwells were combined, added to 500 µL M199 without phenol red (Gibco), and seeded onto glass coverslips coated with 50 µg/mL type I collagen. Cells were allowed to adhere for 10 minutes at 37°C and then non-adherent cells and free dye were removed by rinsing with M199 without phenol red. Coverslips were mounted onto glass slides using M199 without phenol red for live imaging and analysis. The percent of cells containing fluorescently labeled intracellular vacuoles and the number of fluorescently labeled intracellular vacuoles per cell were quantified for each condition (n = 400 cells).

Formation and implantation of tissue constructs

Tissue constructs were prepared and implanted as described,[3] with some modifications. ECs and NHLFs were cultured in EGM-2 for 24 hours prior to embedding. To assemble the tissue constructs, NHLFs (2 x 10^6 cells/mL) and ECs (1 x 10^6 cells/mL) were suspended in a 10 mg/mL fibrinogen solution in serum-free EGM-2. 5% FBS was added and gels were formed in polydimethlysiloxane (PDMS) chambers by mixing 150 µL of the cell-fibrinogen solution with 0.6 U of thrombin (Sigma-Aldrich). Polymerized tissue constructs were submerged in EGM-2 in 12-well plates and cultured overnight. The following day, each tissue construct was implanted into a bluntly dissected subcutaneous pouch on the dorsal surface of an ICR-SCID mouse (Taconic

Farms). For each experiment, two tissue constructs were implanted into each mouse (one per condition) and a total of four animals were used. To stimulate the angiogenic response prior to insertion of the tissue constructs, the mouse tissue lining the inside of the pouch was wounded by gentle scraping using a cytology brush (Fisher Scientific) and 100 µL of EGM-2 was dispensed into the pouch. After 8 days, animals were sacrificed and the tissues and adjacent mouse skin were harvested and fixed in 10% formalin. Sections with a thickness of 5 µm were cut from paraffin-embedded tissues (AML Laboratories) for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical staining was performed as previously described.[3] Tissue sections were stained with mouse monoclonal anti-human CD31 primary antibody (M0823; Dako) diluted 1:200 followed by an HRP-conjugated goat anti-mouse secondary antibody (P0447; Dako) diluted 1:100. The tissue sections were counterstained with hematoxylin and eosin and bright field images covering the entire implant tissue were analyzed using NIH ImageJ software. The total area of each implant tissue was manually traced and converted from pixels to mm^2. The number of vessel lumens per area of implant tissue was then manually quantified.

All images of human tissue sections stained for IFITM1 or vWF and counterstained with hematoxylin were obtained from The Human Protein Atlas, with permission (www.proteinatlas.org).[4]

Immunofluorescence

Angiogenesis assays used for immunofluorescence were performed in Lab-Tek II 4-well chambered borosilicate coverglass dishes (No. 1.0; Thermo Fisher Scientific). The NHLF monolayer was removed from the gels using 5 mg/mL trypsin prior to staining. Cultures were fixed in 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. F-actin was stained using 0.2 µM Texas Red-X phalloidin (Invitrogen) and nuclei were stained with 1 µg/mL DAPI (Sigma-Aldrich).

For immunoflourescent staining of monolayer ECs, siRNA-transfected cells were re-plated into Lab-Tek II 4-well chambered borosilicate coverglass dishes and grown to 100% confluence in EGM-2. Cells were fixed in ice-cold acetone for 15 minutes at -20°C followed by permeabilization with ice-cold methanol for 20 minutes at -20°C. Cells were incubated with mouse monoclonal anti-occludin (4 µg/mL) or rabbit polyclonal anti-claudin-5 (1:100, ab53765; Abcam) primary antibodies followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse (1:400, A-11029; Molecular Probes) or FITC-conjugated goat anti-rabbit (1:100, 111-095-045; Jackson ImmunoResearch) secondary antibodies, respectively.

Permeability Assay

ECs were seeded onto type I collagen-coated (50 $\mu g/cm^2$) 24-well transwell inserts (polyester, 0.4 µm pore size; BD Biosciences) and grown to 100% confluence. FITC-conjugated dextran (1 mg/mL, 40 or 150 kDa; Invitrogen) was added to transwells and at each time point, the fluorescence of media samples from the bottom chambers was measured using a fluorescence microplate reader (Gemini XPS; Molecular Devices) equipped with SoftMax Pro software (Molecular Devices). For each time point, duplicate media samples from each of 4 wells were measured per condition.

Microscopy

For conventional bright field and fluorescence microscopy, an inverted microscope (IX70; Olympus) equipped with the following objectives was used: Plan Semi-Apochromat 10X 0.3 NA objective (Olympus), Plan Semi-Apochromat 4X 0.13 NA objective (Olympus). Images were captured using a SPOT Idea 3.0 megapixel color mosaic camera and Spot acquisition software

(Spot Imaging Solutions). For confocal microscopy, images were captured using an Olympus FV1000 inverted laser scanning confocal microscope (IX81; Olympus) equipped with a Plan Apo 40X 1.3 NA oil objective (Olympus) and FluoView acquisition software (Olympus), or a Nikon Eclipse Ti inverted confocal microscope (Nikon) equipped with a Plan Fluor 40X 1.3 NA DIC oil objective (Nikon), CoolSNAP ES2 CCD camera (Photometrics), and EZ-C1 acquisition software (version 3.91; Nikon). Confocal images obtained were 12-bit (containing 1024×1024 pixels) and four scans were averaged per pixel. Minor adjustments to image brightness and/or contrast were performed using Adobe Photoshop software.

Time-lapse video microscopy

To generate microwells with a glass bottom suitable for imaging, polystyrene cylinders (4.7 mm or 9.5 mm diameter; Scienceware) were secured to the bottom of 33 mm glass bottom tissue culture dishes (No. 1.0; MatTek) using silicone adhesive. Lumenogenesis assays were performed as described above, except that the cell-collagen mix was added at 50 μL per well and the media volume was reduced to 90 μL per well. Angiogenesis assays were performed as described above, except that the fibrinogen/bead suspension was added at 200 μL per well, the media volume was reduced to 250 μL per well, and the number of NHLFs was reduced to 10,000 per well. Cells were imaged using an Axio Vert 200m microscope (Zeiss), Plan-Neofluar 10X 0.3 NA objective (Zeiss) with DIC, a 0.55 NA condenser set to 0.25 NA, and Axiovision software (Zeiss). Dishes were immobilized using clips attached to a custom-milled 6-well aluminum insert (Applied Scientific Instrumentation) that was immobilized in a microtiter tray holder (Frame K-M, Zeiss). A glass lid (CO_2-deckel HM) was applied to the K-M frame and an atmosphere of 5% CO_2 was maintained using a CO_2 controller (PeCon GmbH) with pump setting #3 and reducing valve #6.5. The CO_2 mixture was bubbled through sterile distilled water to maintain humidity. A temperature of 37°C was maintained by enclosing the microscope in a plexiglass chamber (Incubator XL-3, PeCon GmbH) with heating unit ventilation speed #3 and heating intensity #2 (PeCon GmbH). To maintain stable focus, the microscope and incubator were supported on a vibration isolation table (Newport Corporation). Prior to imaging, plastic lids were replaced with lids containing No. 1.0 thickness glass coverslip. Images were captured every 10 minutes for 72 hours for lumenogenesis assays or every 10 minutes for 40 hours for angiogenesis assays using a monochrome CCD camera (AxioCam HRm, Zeiss). Videos were exported from Axiovision and trimmed using QuickTime to remove images taken after contraction and collapse of the collagen gels. Videos were compressed (H264 compression) to less than 10 MB and constrained to 520 x 420 pixels using Prism Video File Converter (v1.88 Intel, NCH Software).

For lumenogenesis assays, the stability of cell-cell contacts was quantified by counting the number of cells that maintained, lost, or never made contact with a neighboring cell during morphogenesis. Three videos per condition were analyzed (n = 68 cells). ECs that underwent apoptosis during the assay were excluded from analysis. Cell numbers were averaged for the three videos and expressed as a percent of the total number of cells quantified for each condition. For angiogenesis assays, the stability of cell-cell contacts was quantified by counting the number of sprouts that maintained or lost cellular contacts during morphogenesis. Two videos per condition were analyzed (n = 19 sprouts).

Sequence alignment and phylogenetic analysis

Human IFITM protein sequence alignment and percent identities were generated using ClustalW2[5,6]. For phylogenetic analysis, we retrieved members of the *IFITM* multigenes from RefSeq database[7] and H-InvDB.[8] A web-based BLASTP analysis[9] was performed using the amino acid sequence of *IFITM1* in human as a query. We searched well-annotated genomic sequences of four mammalian species, human (*Homo sapiens, Hs*), chimp (*Pan troglodytes,*

Pt), mouse (*Mus musculus, Mm*) and rat (*Rattus norvegicus, Rn*). BLASTP-*E*-value, $\leq 10^{-5}$, was used as a cutoff. Then, the obtained result was carefully curated such that alternative isoforms and ambiguously annotated genes (annotated as "similar to —") were excluded. The *PRRT2* gene of the green spotted pufferfish (*Tetraodon nigroviridis, Tn*) was included as an outgroup. Amino acid sequences were aligned using CLUSTALW ver. 1.83.[10] A neighbor-joining tree was inferred from the *p*-distance matrix.[11] Confidential limit of nods was estimated by bootstraps with 10,000 trials, implemented in MEGA ver. 5.[12]

Statistical analysis

All quantifications were performed with the researcher blinded to the conditions and experiments were repeated at least three times (except where indicated) with similar results. Data are reported as mean ± SEM. Differences between experimental groups were analyzed using Student's t-test with *P* values less than 0.05 considered statistically significant.

References

1. Nakatsu MN, Hughes CC. An optimized three-dimensional in vitro model for the analysis of angiogenesis. *Methods Enzymol.* 2008;443:65-82.
2. Koh W, Stratman AN, Sacharidou A, Davis GE. In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. *Methods Enzymol.* 2008;443:83-101.
3. Ghajar CM, Chen X, Harris JW, Suresh V, Hughes CC, Jeon NL, Putnam AJ, George SC. The effect of matrix density on the regulation of 3-D capillary morphogenesis. *Biophys J.* 2008;94:1930-1941.
4. Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S, Wernerus H, Bjorling L, Ponten F. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol.* 2010;28:1248-1250.
5. Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R. A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res.* 2010;38:W695-699.
6. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. *Bioinformatics.* 2007;23:2947-2948.
7. Pruitt KD, Tatusova T, Maglott DR. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* 2007;35:D61-65.
8. Yamasaki C, Murakami K, Fujii Y*, et al.* The H-Invitational Database (H-InvDB), a comprehensive annotation resource for human genes and transcripts. *Nucleic Acids Res.* 2008;36:D793-799.
9. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997;25:3389-3402.
10. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994;22:4673-4680.
11. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 1987;4:406-425.
12. Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform.* 2008;9:299-306.