Interferon-Induced Transmembrane Protein 1 Regulates Endothelial Lumen Formation During Angiogenesis

Stephanie A. Popson, Mary E. Ziegler, Xiaofang Chen, Matthew T. Holderfield, Cameron I. Shaaban, Ashley H. Fong, Katrina M. Welch-Reardon, Jackie Papkoff, Christopher C.W. Hughes

Objective—It is well established that angiogenesis is a complex and coordinated multistep process. However, there remains a lack of information about the genes that regulate individual stages of vessel formation. Here, we aimed to define the role of human interferon-induced transmembrane protein 1 (IFITM1) during blood vessel formation.

Approach and Results—We identified IFITM1 in a microarray screen for genes differentially regulated by endothelial cells (ECs) during an in vitro angiogenesis assay and found that IFITM1 expression was strongly induced as ECs sprouted and formed lumens. We showed by immunohistochemistry that human IFITM1 was expressed by stable blood vessels in multiple organs. siRNA-mediated knockdown of IFITM1 expression spared EC sprouting but completely disrupted lumen formation, in both in vitro and in an in vivo xeno-transplant model. ECs lacking IFITM1 underwent early stages of lumenogenesis (ie, intracellular vacuole formation) but failed to mature or expand lumens. Coimmunoprecipitation studies confirmed occludin as an IFITM1 binding partner in ECs, and immunocytochemistry showed a lack of occludin at endothelial tight junctions in the absence of IFITM1. Finally, time-lapse video microscopy revealed that IFITM1 is required for the formation of stable cell–cell contacts during endothelial lumen formation.

Conclusions—IFITM1 is essential for the formation of functional blood vessels and stabilizes EC–EC interactions during endothelial lumen formation by regulating tight junction assembly. (Arterioscler Thromb Vasc Biol. 2014;34:1011-1019.)

Key Words: angiogenesis modulators ■ blood vessels ■ endothelial cells ■ intercellular junctions

The formation of endothelial cell (EC)–lined blood vessels begins during embryonic development and continues in the adult, both under physiological conditions (eg, growth, wound healing) and in numerous disease states. Whether formed by vasculogenesis (de novo assembly) in the embryo or by angiogenesis (remodeling and sprouting from the pre-existing vasculature), all new vessels must establish a lumen—the defining feature of the vasculature.1

The cellular mechanisms that coordinate EC lumen formation have been described using a variety of in vitro and in vivo models, and although common mechanisms do emerge, some controversy remains. For example, a cord hollowing and cellular rearrangement mechanism, in which the lumen forms de novo between ECs arranged in a cord, was observed in the retina and developing aorta in mice and in the intersegmental and 2 major axial vessels in zebrafish embryos.2–6 In contrast, other groups reported a cell hollowing mechanism, in which ECs form intracellular vacuoles that are exocytosed and fused between neighboring cells to form an intercellular lumen, again, in the developing aorta in mice and in the intersegmental vessels in zebrafish embryos, as well as in cultured ECs within 3-dimensional (3D) gels.7–11 Our data using an in vitro angiogenesis assay suggested that lumen formation might involve a combination of these 2 mechanisms.12 In agreement with this, Wang et al13 reported that lumen formation in the zebrafish intersegmental vessels proceeds via a cord hollowing mechanism driven, in part, by the formation of intracellular vacuoles. The molecular regulators of vascular lumen formation modulate a variety of cellular processes, including cell–cell adhesion, extracellular matrix degradation, polarity, and motility.14 Although this list continues to grow, our understanding of the molecular complexity of endothelial lumen formation remains in its infancy.

Human interferon-induced transmembrane protein 1 (IFITM1), which was originally identified as a downstream target of interferon stimulation,15 belongs to the IFITM gene family, along with IFITM2, IFITM3, IFITM10, and bone-specific IFITM5/BRIL.16,17 IFITM1 exhibits a diverse range of cellular...
functions that are cell-type and context dependent, including proliferation, adhesion, and cellular resistance to viral infection.\textsuperscript{18,19} The 7 \textit{Ifitm/fragilis} genes in mice are homologs, but not orthologs, of the human genes. Only a single ancestral gene was passed to each species, where it then duplicated independently within each lineage. Thus, the gene families may have acquired completely independent functions.\textsuperscript{20}

More than 20 years ago, a study reported that blood vessel ECs in several adult organs express human IFITM1.\textsuperscript{21} Moreover, IFITM1 expression is induced in cultured ECs in response to interferon\textsuperscript{22} and \textit{IFITM1} may be a pan-endothelial marker.\textsuperscript{23} Surprisingly, however, the function of IFITM1 in ECs has not been investigated. Therefore, we asked whether IFITM1 regulates the formation of functional EC-lined blood vessels. To determine the cellular mechanisms of IFITM1 function in ECs, we used RNAi and 3D in vitro models of vessel formation. We examined the role of human IFITM1 during vessel formation in vivo using a murine xeno-transplant vascular bed model\textsuperscript{24}, a strategy to avoid inferring gene function by comparing nonorthologous human and mouse \textit{IFITM/fragilis} genes.

### Results

**Endothelial IFITM1 Expression Is Regulated During Angiogenesis In Vitro**

We previously developed an in vitro angiogenesis assay, in which human ECs sprout from the surface of beads embedded in fibrin gels to form microvessels.\textsuperscript{12} In this model, ECs undergo a series of coordinated morphological changes that recapitulate the critical stages of in vivo angiogenesis, including proliferation, migration, sprouting, branching, and lumen formation (Figure 1A). Using this assay, we performed microarray analyses to examine temporal gene expression changes in ECs actively undergoing tube formation (unpublished data). Notably, we identified IFITM1 as being strongly induced on day 4, correlating with the onset of lumen formation, with expression peaking on day 6 (Figure 1B). This expression pattern was confirmed independently using quantitative reverse transcription polymerase chain reaction (Figure 1C).\textsuperscript{25} In addition, Western blot analysis revealed a gradual and cumulative increase of IFITM1 protein in ECs during the course of 10 days (Figure 1D). To determine the expression of IFITM1 by ECs in vivo, we examined normal human tissue immunohistochemically stained with an IFITM1 antibody. As shown in Figure 1E, IFITM1 was expressed by blood vessel ECs in the bladder, brain, and stomach and exhibited a staining pattern...

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>IFITM1</td>
<td>interferon-induced transmembrane protein 1</td>
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<td>TJ</td>
<td>tight junction</td>
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**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

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**Figure 1.** Interferon-induced transmembrane protein 1 (IFITM1) is regulated by endothelial cells (ECs) during in vitro angiogenesis and expressed by blood vessels in vivo. A, Representative images showing morphological progression of developing EC sprouts during in vitro angiogenesis for 10 days. Asterisks indicate vessel lumens. B and C, ECs were harvested from angiogenesis assays at the indicated times and IFITM1 mRNA expression was analyzed by microarray (B) or quantitative reverse transcription polymerase chain reaction (C).\textsuperscript{25} Data are represented as fold change over day 0±SEM (n=3). *P<0.05. D, ECs were harvested from angiogenesis assays at the indicated times and IFITM1 protein was examined by Western blot. Blots were probed for β-actin as a loading control. Densitometry values normalized to β-actin are expressed as fold change over day 0±SEM (n=3). *P<0.05, **P<0.005. E, Human tissue sections were immunohistochemically stained for IFITM1 (left) or von Willebrand factor (right) and counterstained with hematoxylin. Arrows point to blood vessels. Images are from The Human Protein Atlas (http://www.proteinatlas.org/), with permission of the publisher.
similar to the EC marker, von Willebrand factor. Thus, endothelial IFITM1 expression correlates with vessel maturation: it is induced by ECs during the maturation stages of angiogenesis in vitro and is stably expressed by quiescent microvascular ECs in vivo.

**IFITM1 Regulates EC Lumen Formation During Angiogenesis In Vitro**

To elucidate the function of IFITM1 in ECs, we used RNAi to knock down expression and then examined the ability of the cells to undergo morphogenetic events during angiogenesis. Human IFITM1 exhibits ≈70% to 80% mRNA sequence identity with 2 of its family members, IFITM2 and IFITM3 (Figure IA in the online-only Data Supplement). Thus, we confirmed the specificity of the siRNA for IFITM1 transcripts. As shown in Figure IB in the online-only Data Supplement, transfection of ECs with IFITM1 siRNA had no significant effect on expression of either IFITM2 or IFITM3 mRNA. In contrast, IFITM1 mRNA was significantly reduced (>95%), as was protein expression, as confirmed by Western blot (Figure 2A and 2B). The residual protein band observed after IFITM1 siRNA treatment was not IFITM1 protein, but rather IFITM3 protein, which was also detected by the IFITM1 antibody, as demonstrated by the addition of IFITM3 siRNA (Figure 2B, compare lanes 2 and 3). Knockdown of IFITM1 was persistent, with inhibition still >80% at 7 days post transfection (Figure IC in the online-only Data Supplement).

When we tested the effect of IFITM1 knockdown in ECs during angiogenesis in vitro, we found that although there was no significant effect on sprouting, EC lumen formation was almost completely inhibited (Figure 2C–2E). Similar results were obtained using an independent IFITM1 siRNA (Figure IIA–IIE in the online-only Data Supplement). Next, to better visualize EC lumens within fibrin matrices, control and IFITM1 siRNA-treated cultures were stained for F-actin and examined using confocal microscopy. Control vessels contained a continuous lumen surrounded by a single layer of ECs along the length of the vessel (Figure 2F, left). In contrast, IFITM1 knockdown vessels lacked a lumen and instead were organized into cords of ECs (Figure 2F, right). Collectively, these results demonstrate that IFITM1 expression is required for EC lumen formation during angiogenesis in vitro.

To confirm these initial findings, we examined the effects of IFITM1 knockdown on EC lumens using a second in vitro assay. In this system, ECs are suspended as single cells within a 3D collagen matrix and stimulated to undergo vacuole and lumen formation.11 As shown in Figure 3A and 3B, ECs transfected with control siRNA formed large multicellular lumens within the collagen matrix. In contrast, knockdown of IFITM1 permitted only the formation of intracellular vacuoles and rudimentary intercellular lumens (Figure 3A and 3B). IFITM1 knockdown using a second IFITM1 siRNA produced similar results (Figure IIF and IIG in the online-only Data Supplement). Thus, IFITM1 is required for EC lumen formation within collagen matrices, consistent with our observations during angiogenesis within fibrin matrices (Figure 2).

Finally, we performed rescue experiments to determine whether re-expressing IFITM1 in IFITM1-knockdown ECs could restore lumen formation. We designed an IFITM1

![Figure 2. Interferon-induced transmembrane protein 1 (IFITM1) is required for endothelial cell (EC) lumen formation during angiogenesis in vitro. A, ECs were transfected with control or IFITM1 siRNAs and knockdown of IFITM1 mRNA was assessed by quantitative reverse transcription polymerase chain reaction 24 hours later. Data are represented as percentage of control expression (set to 100±SEM (n=3). B, ECs were transfected with indicated siRNAs and knockdown of IFITM1 protein was examined by Western blot 72 hours later. Residual signal detected after IFITM1 siRNA treatment was not IFITM1 protein, but rather IFITM3 protein (compare lanes 2 and 3). Blots were probed for β-actin as a loading control. C, ECs transfected with control or IFITM1 siRNA were seeded into angiogenesis assays for 6 days and then photographed. Arrows indicate vessels containing a continuous intercellular lumen. D and E, Assays described in C were analyzed for the number of sprouts (D) and percentage of lumenerized sprouts (E). Values are means±SEM (n=3). **P<0.005. F, Assays described in C were stained for F-actin (phalloidin, red) and nuclei (4′,6-diamidino-2-phenylindole, blue) and visualized using confocal fluorescence microscopy. Images of stained vessels were captured at 3 focal planes (top, center, and bottom). Enlarged images of center cross sections (dotted white boxes) are shown in the bottom. White asterisks indicate vessel lumens.](http://atvb.ahajournals.org/|

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siRNA (IFITM1 #2) targeting the 3′ untranslated region of IFITM1 mRNA and generated a retrovirus encoding an N-terminally FLAG-tagged IFITM1 protein lacking the 3′ siRNA-targeted sequence. Transfection of ECs with IFITM1 #2 siRNA knocked down both IFITM1 mRNA and protein (Figure IIA and IIB in the online-only Data Supplement) and as mentioned above, impaired EC lumen formation in vitro (Figure IIC–IIG in the online-only Data Supplement). Expression of the FLAG-IFITM1 protein in transduced ECs was confirmed by Western blot using a FLAG-specific antibody (Figure 3C). As shown in Figure 3D and 3E, the FLAG-IFITM1 retrovirus significantly rescued lumen formation in IFITM1 #2 siRNA-treated ECs, compared with the control green fluorescent protein retrovirus. Specifically, large multicellular lumens were observed in the presence of the FLAG-IFITM1 retrovirus that morphologically resembled control cultures (Figure 3D, compare left and right panels). Thus, stable re-expression of IFITM1 protein restores lumen formation in IFITM1-knockdown ECs. We conclude from this series of experiments that IFITM1 regulates EC lumenogenesis in both fibrin and collagen matrices in vitro.

Figure 3. Retroviral delivery of interferon-induced transmembrane protein 1 (IFITM1) rescues the IFITM1-knockdown phenotype. A and B, Endothelial cells (ECs) transfected with control or IFITM1 siRNAs were seeded into lumenogenesis assays and allowed to undergo morphogenesis for 48 hours. A, Images from a representative experiment are shown. Arrows point to large multicellular lumens in control cultures. Arrowheads indicate small intercellular lumens in IFITM1-knockdown cultures. B, Assays were quantified for the mean lumenal area±SEM (n=3). *P<0.05. C, ECs were transduced with the indicated retroviruses and ectopically expressed FLAG-IFITM1 protein was examined by Western blot 72 hours later using a FLAG antibody. Blots were probed for β-actin as a loading control. D and E, ECs were transduced with retroviruses encoding green fluorescent protein or FLAG-IFITM1, transfected with siRNAs as indicated, and seeded into lumenogenesis assays for 48 hours. D, Images from a representative experiment are shown. Arrows point to large multicellular lumens. Arrowheads indicate small intercellular lumens. E, Assays were quantified for mean lumenal area±SEM. **P<0.005.

Human IFITM1 Is Required for Blood Vessel Formation In Vivo

Our knockdown studies demonstrate that IFITM1 is required for endothelial lumen formation in vitro. Therefore, we next wanted to test whether IFITM1 might similarly regulate vessel formation in vivo. There are 7 Ifitm/fragilis genes in mice. However, phylogenetic analyses performed by us and others indicate that IFITM/fragilis genes in human and mouse are not orthologs, but rather they are homologs (Figure III in the online-only Data Supplement).20 The IFITM/fragilis gene family in each species arose from a single common ancestral gene that was subsequently duplicated independently within each lineage. Thus, it is not possible to directly compare individual IFITM/fragilis gene functions between the 2 species. Therefore, we turned to a xeno-transplant vascular bed model, whereby human ECs suspended in a fibrin gel form microvessels that anastomose with the host vasculature when implanted under the skin of a mouse (Figure 4A).24 As shown in Figure 4B, control siRNA-treated ECs organized into microvessels within the implant tissue and formed functional anastomoses with host vessels, as evidenced by the presence of mouse erythrocytes within the human vessels. In sharp contrast, IFITM1-knockdown ECs failed to form functional vessels (Figure 4C). Instead, only rudimentary vessel-like EC cords lacking a distinct lumen were observed (Figure 4C and 4D). Notably, these nonperfused vessels were unable to support the robust stromal cell growth observed in control tissues (Figure 4A–4C). The failure to form lumenzized structures was not because of a defect in cell migration, as control and IFITM1-knockdown cells showed similar migratory rates in 3D collagen gels (control, 0.27±0.04 μm/min; IFITM1-knockdown, 0.29±0.05 μm/min). These transplant studies are entirely consistent with our in vitro data and demonstrate that IFITM1 expression is also required for human vessels to form lumens in vivo.
IFITM1 Regulates Endothelial Lumen Formation

EC lumen formation has been proposed to occur via a step-wise process, beginning with the formation of intracellular pinocytic vacuoles, followed by intracellular vacuole fusion and ending with intercellular coalescence of neighboring EC vacuoles. Expansion and remodeling then follows to form a continuous vessel lumen. To determine whether IFITM1 is required for pinocytic vacuole formation, we examined the process of lumen formation in collagen matrices in the presence of fluorescein isothiocyanate-dextran. During the course of 4 hours, newly formed pinocytic vacuoles become fluorescently labeled and could be easily visualized. Interestingly, siRNA-mediated suppression of IFITM1 had no effect on the ability of ECs to form intracellular vacuoles. Next, we examined EC vacuole formation during angiogenesis within fibrin matrices by staining for F-actin, which associates with EC vacuole membranes during lumen formation. Consistent with our observations in collagen matrices, several distinct intracellular vacuoles were present in ECs in fibrin gels even in the absence of IFITM1 expression. Moreover, we also found evidence of incomplete intercellular vacuole fusions between neighboring ECs within individual sprouts. Collectively, these studies demonstrate that IFITM1 expression is dispensable for EC intracellular vacuole formation and suggest that IFITM1 regulates a subsequent morphological event during EC lumen formation.

IFITM1 Regulates Junctional Stability During EC Lumen Formation

A critical step during EC vessel and lumen maturation is stabilizing EC–EC junctions. As IFITM1 was shown recently to associate with the tight junction (TJ) protein occludin in hepatocytes, we reasoned that the failure of IFITM1-knockdown ECs to form mature lumens might be explained by their inability to assemble stable TJ adhesion complexes. Therefore, we first confirmed the physical interaction of IFITM1 and occludin proteins in ECs using coimmunoprecipitation. Next, we examined the effects of IFITM1 knockdown on occludin localization in confluent EC monolayers and found that occludin was almost completely absent from TJs in IFITM1 siRNA-treated ECs. Knockdown of IFITM1 also caused aberrant cytosolic accumulation of a second TJ protein, claudin-5, providing further evidence for a defect in TJ formation. In addition, IFITM1-knockdown EC monolayers exhibited impaired TJ barrier function, as determined by an increase in paracellular permeability. Finally, to determine whether endothelial junctional stability was disrupted in IFITM1-knockdown ECs during lumen formation, we used time-lapse video microscopy. ECs transfected with either of the 2 control siRNAs established initial intercellular interactions within the 3D collagen matrix that remained relatively stable over time to facilitate the formation, remodeling, and expansion of multicellular lumens. In sharp contrast, ECs transfected with either of the 2 IFITM1 siRNAs formed unstable, dynamic intercellular interactions.
Cycling through phases of initiation followed by disassembly (Figure 6D and 6E). Junctional stability was similarly disrupted in IFITM1-knockdown ECs during sprouting angiogenesis in fibrin gels (Figure V in the online-only Data Supplement and data not shown). Collectively, these data demonstrate that IFITM1 is required for the proper localization of occludin and claudin-5 in ECs and suggest that IFITM1 may regulate endothelial TJ stability during lumen formation.

Discussion

In the present work, we define a novel role for IFITM1 in endothelial lumenogenesis during blood vessel formation. Using an siRNA approach to knock down IFITM1 expression, we developed a clear understanding of the cellular mechanisms underlying IFITM1 function in ECs during vascular morphogenesis. Although IFITM1 was dispensable for EC sprouting and migration within 3D matrices, it was absolutely required for multicellular lumen formation and subsequent vessel maturation. IFITM1-knockdown ECs proceeded normally through the early stages of lumen formation, which includes the formation of pinocytotic vacuoles and their subsequent intracellular fusion, whereas the later phase of intercellular vacuole fusion to yield multicellular lumens invariably failed, apparently because of mislocalization of the TJ proteins occludin and claudin-5.

We think that our observations using 2 different in vitro assays reflect the same defect—an inability to stabilize junctions. This was most obvious in collagen gel lumenogenesis assays, in which ECs begin as single cell suspensions in the gel; we observed IFITM1-knockdown ECs coming together, forming temporary contacts, and then separating again before stable intercellular lumens could form. Interestingly, in fibrin gel angiogenesis assays, in which cells are in contact from the beginning of the assay, we did not see IFITM1-knockdown ECs migrating away from one another. Rather, we noted that cords of connected ECs migrated (sprouted) away from the bead, but never underwent intercellular lumen formation. Although we do not yet have a clear explanation for this difference, it may relate to the local microenvironment, which in the sprouting assay contains fibroblast-derived factors. Indeed, we previously
demonstrated that fibroblast-derived matricellular proteins play a critical role in lumen formation, partially by increasing gel stiffness. Thus, it may be that this modified matrix constrains ECs during junctional remodeling, whereas in the absence of fibroblasts, such as in the collagen gel lumenogenesis assay, ECs more readily migrate away from each other. In support of this hypothesis, we found that in our in vivo model, where fibroblasts are present, cords of IFITM1-knockdown ECs were present, and these cords lacked a lumen.

Further support for the idea that IFITM1 regulates junctional assembly and stability comes from our observation that IFITM1 interacts with the TJ protein occludin in ECs, consistent with a recent report in hepatocytes. Importantly, we demonstrated that occludin failed to localize to TJs in the absence of IFITM1 expression. Occludin knockout mice, however, are viable and exhibit normal TJ morphology, with no reported vascular defects. These discrepancies may reflect inherent differences between mice and humans or might suggest that IFITM1 knockdown has additional effects on endothelial TJ assembly. In agreement with the latter, we also observed aberrant localization of a second TJ protein, claudin-5, in IFITM1-knockdown ECs.

Although our data clearly demonstrate dysregulated localization of occludin and claudin-5 in the absence of IFITM1 expression in ECs, the precise molecular mechanisms underlying IFITM1 regulation of TJ assembly and stability remain elusive. One potential mechanism might involve a role for IFITM1 in coordinating the recycling of internalized TJ
proteins back to the plasma membrane during TJ remodeling. Indeed, IFITM proteins function in the endosomal pathway to inhibit viral infection and internalized occludin and claudin-5 are returned to the plasma membrane from recycling endosomes during TJ remodeling in brain ECs. Alternatively, IFITM1 protein may itself be a critical component of the endothelial TJ complex, as IFITM1 has been shown to localize to hepatic TJs. Distinguishing between these and other possibilities will be the focus of future studies.

As noted above, our in vivo data using a xeno-transplant vascular bed model were consistent with our in vitro observations: in the absence of IFITM1 expression, implanted human ECs failed to form functional, mature, lumenized vessels. This result is further supported by our observation that IFITM1 is expressed by quiescent blood vessel ECs in multiple human tissues, in agreement with previous reports. Taken together, these data suggest that IFITM1 is required for both the formation and the maintenance of blood vessel lumens.

The use of human ECs in these studies proved critical, as our phylogenetic analysis revealed that although the human and mouse IFITM1/fragilis gene family, they are not orthologs—a single ancestral gene (predicted to be IFITM1/BRIL) was passed to each lineage where it subsequently underwent independent duplications, a conclusion also drawn by others. Thus, manipulation of mouse fragilis genes could not be used to reliably investigate the function of the human IFITM1 gene. In agreement with this, our studies silencing human IFITM1 in ECs demonstrated that the expression of IFITM2 and IFITM3 was not sufficient to functionally compensate for the loss of IFITM1, whereas mice carrying a deletion of either the entire fragilis locus on chromosome 7 (encompassing 5 fragilis family members) or only the Ifitm3/fragilis gene had no discernable phenotype, despite a previously defined role in murine embryonic development, suggesting functional redundancy and compensation within the mouse fragilis gene family.

Our studies in vitro and in vivo indicate that IFITM1 regulates EC lumen formation within a diverse range of complex extracellular matrices, suggesting that the function of IFITM1 is not matrix, or by implication, integrin specific. These findings also highlight the potential relevance of IFITM1 function to vessel formation, both within physiological settings, in which type I collagen dominates the interstitial matrix, and pathological settings, which are characterized by a provisional matrix rich in vitronectin, fibronectin, and fibrin. Our results also implicate IFITM1 in diseases such as diabetes mellitus, stroke, and hypoxia/aglycemia, in which vessel barrier function is disrupted in association with occludin downregulation.

In summary, our data define IFITM1 as a novel regulator of EC lumen formation during vascular morphogenesis. We describe a mechanism by which IFITM1 regulates the expansion and maturation of nascent EC lumens into multicellular tubular networks by regulating either the formation, stability or formation and stability, of endothelial TJs. Thus, IFITM1 may prove to be a useful therapeutic target in settings of pathological angiogenesis.

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Disclosures
None.

References


**Significance**

The ability to make multicellular tubes is restricted to cells of the epithelial lineage, including endothelial cells, and is a process critical to the formation of a functional vascular network. Our study identifies interferon-induced transmembrane protein 1 as a novel regulator of endothelial tube (lumen) formation during angiogenesis. Using a combination of in vitro and in vivo models, we demonstrate that interferon-induced transmembrane protein 1 functions to promote maturation of nascent lumens by regulating the assembly and stability of intercellular tight junction adhesion complexes. These findings suggest that interferon-induced transmembrane protein 1 may be a potential therapeutic target for pathological angiogenesis and implicates interferon-induced transmembrane protein 1 in diseases associated with disrupted tight junctions.
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Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/03/06/ATVBAHA.114.303352.DC1
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% CO₂. Normal human lung fibroblasts (NHLFs; Lonza) were routinely grown in M199 supplemented with 10% FBS at 37°C and 5% CO₂. 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'-UAAUAUGGUAGCUGACAGAGCC-3' (antisense); IFITM3, 5'-UGAAUCACACUGUCCAAAACCUCUU-3' (sense) and 5'-AAGAAGGUUUGACUGUAAUCA-3' (antisense). Silencer select siRNAs to human IFITM1 were purchased from Ambion and the sequences are as follows: IFITM1 #2, 5'-GCCUAUAGCCUGCAACCUtt-3' (sense) and 5'-AAGGUUGCAGGCUAUGGCgg-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% CO₂. 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'-CGTCCAGGCCAGCCAGATAGT-3' (upper) and 5'-GCAGGGCGAGAATGAAGATAG-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. 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 luminal 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 polyacrylamide 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. Briefly, ECs suspended in rat-tail collagen type I matrices (3.75 mg/mL) at a concentration of 6 x 10⁵ 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 µm², 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, 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 10⁶ cells/mL) and ECs (1 x 10⁶ 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 polydimethylsiloxane (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).
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. 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 mm². 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).

**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 immunofluorescent 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 µg/cm²) 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.
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; Scienteware) 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 (CO2-deckel HM) was applied to the K-M frame and an atmosphere of 5% CO2 was maintained using a CO2 controller (PeCon GmbH) with pump setting #3 and reducing valve #6.5. The CO2 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. For phylogenetic analysis, we retrieved members of the IFITM multigenes from RefSeq database and H-InvDB. A web-based BLASTP analysis 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, ≤ 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. A neighbor-joining tree was inferred from the p-distance matrix. Confidential limit of nods was estimated by bootstraps with 10,000 trials, implemented in MEGA ver. 5.

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

SUPPLEMENTAL DATA

Figure S1. siRNA-mediated knockdown of IFITM1 expression in ECs is efficient and specific. (A) Amino acid sequence alignment of human IFITM1, IFITM2, and IFITM3 proteins. Amino acid residue substitutions are shown in grey. (B) ECs were transfected with control or IFITM1 siRNAs and examined by qRT-PCR 24 hours later for expression of IFITM1, IFITM2, and IFITM3. Data are represented as percent of control expression (set to 100)±SEM for each gene (n=3). (C) ECs were transfected with control or IFITM1 siRNAs and knockdown of IFITM1 mRNA expression was assessed by qRT-PCR at 3 and 7 days post-transfection. Data are represented as percent of control expression (set to 100)±SEM for each time point (n=3).
Figure SII. Knockdown of IFITM1 using an independent siRNA confirmed requirement for IFITM1 during EC lumen formation. (A) ECs were transfected with control #2 or IFITM1 #2 siRNAs and examined by qRT-PCR 48 hours later for expression of IFITM1, IFITM2, and IFITM3. The IFITM1 #2 siRNA was specific for IFITM1 and did not significantly affect the expression levels of IFITM2 or IFITM3. Data are represented as percent of control expression (set to 100)±SEM for each gene (n=3). (B) ECs were transfected with control #2 or IFITM1 #2 siRNAs and knockdown of IFITM1 protein was examined by western blot 72 hours later. Blots were probed for β-actin as a loading control. (C-E) ECs transfected with control #2 or IFITM1 #2 siRNAs were seeded into angiogenesis assays and analyzed for the number of sprouts (D) and percentage of lumenized sprouts (E) 6 days later. Values are means±SEM (n=3). *P<0.05. Images from a representative experiment are shown (C). Arrows indicate vessels containing a continuous intercellular lumen. Scale bars, 100 µm. (F-G) ECs transfected with control #2 or IFITM1 #2 siRNAs were seeded into lumenogenesis assays and fixed 48 hours later. Representative images of toluidine-stained cultures are shown. Arrows point to large multicellular lumens in control cultures (top panel). Arrowheads indicate small intercellular lumen structures in IFITM1 knockdown cultures (bottom panel). Scale bars, 50 µm. (G) Assays were quantified and data are shown as the mean lumenal area±SEM (n=3). *P<0.05.
Figure SIII. Phylogenetic tree for amino acid sequences of the *IFITM* gene family. Bootstrap values ≥ 50% are shown. Scale bar shows *p*-distance of amino acid sequences. *Hs*, *Homo sapiens*; *Pt*, *Pan troglodytes*; *Mm*, *Mus musculus*; *Rn*, *Rattus norvegicus*; *Tn*, *Tetraodon nigroviridis* (outgroup).
Figure SIV. Loss of IFITM1 causes aberrant cytosolic accumulation of claudin-5 protein. (A) ECs transfected with control #2 or IFITM1 #2 siRNAs were immunostained for claudin-5 (green) and nuclei (DAPI, blue). Scale bars, 10 µm. (B) ECs were transfected with control #2 or IFITM1 #2 siRNAs and claudin-5 protein expression was examined by western blot 72 hours later. Blots were probed for β-actin as a loading control. Densitometry values normalized to β-actin are expressed as percent of control±SEM (n=4). *P<0.05.
Figure SV. IFITM1 regulates junctional stability during angiogenesis in vitro. ECs transfected with the indicated siRNAs were seeded into angiogenesis assays and examined by time-lapse video microscopy on day 5. Videos were analyzed for the stability of EC-EC contacts by quantifying the number of sprouts that maintained or lost cellular contacts, expressed as a percent of the total number of sprouts quantified for each condition±SEM (n=2). *P<0.05.
Video S1. ECs transfected with control siRNA were seeded into lumenogenesis assays and examined using time-lapse video microscopy. Images were taken every 10 minutes for 69 hours, after which the collagen gels contracted and collapsed. Movies are shown at a speed of 18 frames per second.

Video SII. ECs transfected with control #2 siRNA were seeded into lumenogenesis assays and examined using time-lapse video microscopy. Images were taken every 10 minutes for 42 hours, after which the collagen gels contracted and collapsed. Movies are shown at a speed of 18 frames per second.

Video SIII. ECs transfected with IFITM1 siRNA were seeded into lumenogenesis assays and examined using time-lapse video microscopy. Images were taken every 10 minutes for 69 hours, after which the collagen gels contracted and collapsed. Movies are shown at a speed of 18 frames per second.

Video SIV. ECs transfected with IFITM1 #2 siRNA were seeded into lumenogenesis assays and examined using time-lapse video microscopy. Images were taken every 10 minutes for 42 hours, after which the collagen gels contracted and collapsed. Movies are shown at a speed of 18 frames per second.