

EGFL7 Mediates BMP9-Induced Sprouting Angiogenesis of Endothelial Cells Derived from Human Embryonic Stem Cells

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SUMMARY

Human embryonic stem cells (hESCs) are instrumental in characterizing the molecular mechanisms of human vascular development and disease. Bone morphogenetic proteins (BMPs) play a pivotal role in cardiovascular development in mice, but their importance for vascular cells derived from hESCs has not yet been fully explored. Here, we demonstrate that BMP9 promotes, via its receptor ALK1 and SMAD1/5 activation, sprouting angiogenesis of hESC-derived endothelial cells. We show that the secreted angiogenic factor epidermal growth factor-like domain 7 (EGFL7) is a downstream target of BMP9-SMAD1/5-mediated signaling, and that EGFL7 promotes expansion of endothelium via interference with NOTCH signaling, activation of ERK, and remodeling of the extracellular matrix. CRISPR/Cas9-mediated deletion of *EGFL7* highlights the critical role of EGFL7 in BMP9-induced endothelial sprouting and the promotion of angiogenesis. Our study illustrates the complex role of the BMP family in orchestrating hESC vascular development and endothelial sprouting.

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent and therefore offer opportunities to investigate early human development, model genetic diseases, and provide approaches to regenerative medicine. Thorough understanding of vascular differentiation of hESCs will yield new insights into how human vasculature develops and regenerates and lead to improved methods for producing vascular cells for transplantation. Gene ablation in mice has shown that the transforming growth factor β (TGF- β) family, which also includes bone morphogenetic proteins (BMPs), nodal, and activin, plays a fundamental role in mesodermal development and vascular commitment. Mutations in Endoglin and ALK1 have been linked to a human vascular disorder, hereditary hemorrhagic telangiectasia (HHT1 and HHT2, respectively), often resulting in arteriovenous malformation (AVM) (Larrivee et al., 2012; Lebrin et al., 2010). Also, mutations in the BMP type II receptor gene are responsible for pulmonary arterial hypertension (PAH) (Morrell et al., 2016).

TGF- β , activin, and nodal all activate SMAD2/3 to promote hESC self-renewal (Vallier et al., 2005). BMPs activate SMAD1/5, which upregulate the expression of ID proteins (inhibitors of DNA) that in turn inhibit bHLH transcription factors (Hollnagel et al., 1999). Data on the role of the BMP family in mediating the vascular commitment of hESCs is sparse. Vascular cells have a mesoderm origin. BMP4 induces mesodermal differentiation and patterning and promotes epithelial to mesenchymal transition (EMT) in

hESCs in part via SLUG and MSX2 (Richter et al., 2014). It is well known that vascular endothelial growth factor (VEGF) also plays a pivotal role in vascular differentiation. Vascular expansion of hESC-derived endothelial cells (ECs) can be induced by inhibition of the TGF- β /SMAD2/3 pathway (James et al., 2010). The BMP9/ALK1/SMAD1/5 pathway has been studied intensively in ECs and in mouse ESC-derived ECs (reviewed in Goumans et al., 2017). The fundamental mechanism by which BMP9/ALK1 signaling can induce or inhibit angiogenesis is, however, still unclear and presumably context dependent. This pathway has so far not been studied in the process of differentiation of hESC toward vascular cells.

The BMP effectors SMAD1/5 have a pivotal role in stalk cell competence by regulating EC migration and activating the target genes of the NOTCH intracellular domain (NICD) in stalk cells in mice (Moya et al., 2012). In addition, ALK1 signaling inhibits angiogenesis in mice by cooperating with the NOTCH pathway (Larrivee et al., 2012). The molecular mechanism driving differential expression of target genes in tip and stalk cells remains unclear although several NICD-dependent genes are also BMP-induced SMAD1/5 targets as shown by chromatin immunoprecipitation sequencing (ChIP-seq) studies in human ECs (Morikawa et al., 2011). ID proteins are targets of BMP-SMAD signaling that play a crucial role in determining the two EC types during the angiogenic phase (Itoh et al., 2004; Moya et al., 2012).

Epidermal growth factor-like domain 7 (EGFL7) is a secreted angiogenic factor that is expressed by and acts

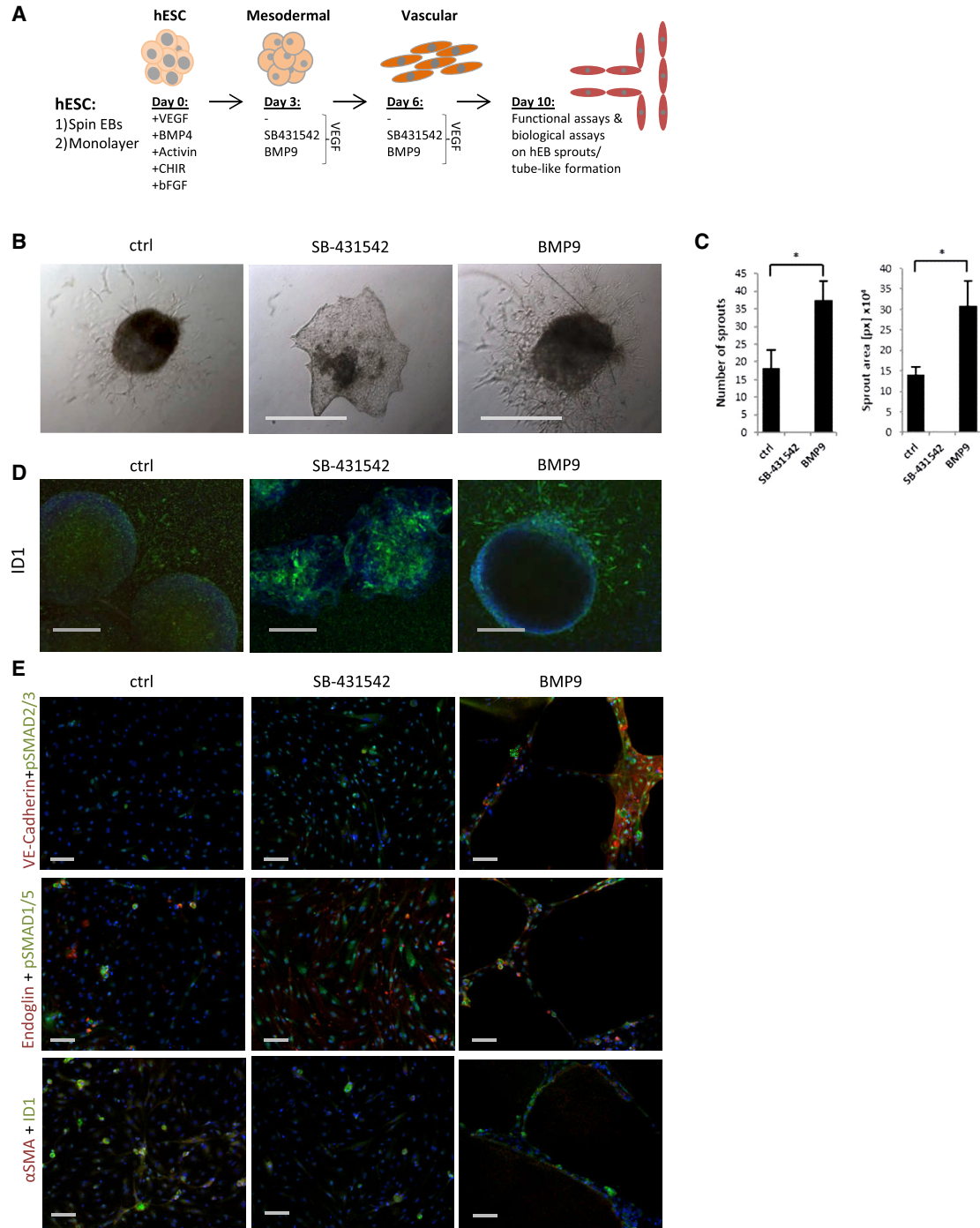


Figure 1. Expansion and Sprouting of hESC-ECs Is Induced by BMP9 Signaling

(A) Schematic workflow of hESC-EC differentiation using (1) spin EBs and (2) monolayer.

(B) EBs generated from hESC were treated according to the protocol described in (A) with a special focus on BMP9 and SB-431542 (ALK4/5/7 inhibitor) treatment. VEGF, control. After 9 days EBs were embedded in collagen I and evaluated 24 h later. Scale bar, 50 μ m.

(C) Quantification of the sprouts by Wimasis (www.wimasis.com) for both length and area of the sprouts from three different experiments. The error bars represent SD. * $p < 0.05$.

(D) Nine-day-old EBs embedded in collagen I were stained with antibody against ID1. Scale bar, 300 μ m.

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on ECs. Expression of EGFL7 is highest in proliferating vasculature and regulates tubulogenesis in zebrafish and vascular patterning and integrity in mice (Nichol and Stuhlmann, 2012). Interestingly, microRNA-126, an EC-specific miRNA, resides in intron 7 of the *EGFL7* gene and regulates vascular integrity via the VEGF regulators SPRED1 and PIK3R2 (Fish et al., 2008; Wang et al., 2008). Ectopic EGFL7 interacts with the extracellular domain of NOTCH and as a result, functions as an antagonist of NOTCH activation (Schmidt et al., 2009). Here, we used hESCs as a model for human vascular commitment to study the underlying molecular mechanisms of BMP9 in vascular development and disease. We demonstrate that BMP9/ALK1/SMAD1/5-induced endothelial sprouting critically depends on EGFL7. We link the BMP signaling pathway for the first time with EGFL7.

RESULTS

BMP9 Induces Expansion and Sprouting of hESC-Derived Vascular Cells

Vascular differentiation of hESCs was obtained in cells that were either forced by centrifugation to aggregate into embryoid bodies (EBs) or in monolayer cultures (Figure 1A). To induce mesodermal differentiation, basic fibroblast growth factor (bFGF), BMP4, ActivinA, the GSK3 inhibitor CHIR99021, and VEGF₁₆₅ were added to the hESCs. Three days later, vascular expansion of mesodermal cells was induced with VEGF alone or supplemented with either SB-431542 (ALK4/5/7 inhibitor) or BMP9 under VEGF conditions. The specificity of SB-431542 was shown by downregulation of *SMAD7* and *PAI-1* (Figures S1A and S1B). To study the effect of BMP9 on the properties of the hESC-ECs, EB sprouting was induced in collagen I and evaluated on day 10; sprouting induced by only VEGF was modest. Interestingly, SB-431542-treated EBs formed sheet-like structures that were unable to invade the collagen, whereas BMP9-treated EBs underwent extensive sprouting (Figure 1B and Videos S1, S2, and S3). The total sprout length and the total area covered by the sprouts was increased by 50%, respectively, compared with the VEGF control condition (Figure 1C). ID1 is a downstream target of BMP-SMAD1/5 signaling and promotes angiogenesis (Lyden et al., 1999; Valdimarsdottir et al., 2002). ID1 expression was investigated in endothelial outgrowth of hEBs. BMP9 treatment of hEBs upregulated ID1 specifically in the sprouts (Figure 1D). ID1 expression was elevated in

SB-431542-treated cells compared with control cells, but those were unable to invade the collagen, which may be due to an increase in stalk cells at the expense of tip cells.

Subsequently, CD31⁺ cells were used in a tube-like formation assay on Matrigel-coated chamber slides. BMP9 treatment resulted in enhanced tube-like formation compared with untreated and SB-431542-treated CD31⁺ cells (Figure 1E). Consistent with the increased tube-like formation following BMP9 treatment, these cells were also enriched for the endothelial markers VE-Cadherin and Endoglin, whereas the smooth muscle differentiation marker α SMA was undetectable. Taken together, BMP9 stimulation promotes tube formation and angiogenic sprouting in hESC-derived ECs.

ALK1 Mediates the BMP9-Induced Sprouting of ECs Derived from hESCs

The high-affinity receptor for BMP9 is the endothelial-specific ALK1 receptor (David et al., 2007), which becomes expressed at day 3 during hESC differentiation (Poon et al., 2006). To study whether BMP9-induced hESC-derived endothelial sprouting is dependent on ALK1, we used the neutralizing anti-human ALK1 antibody PF-03446962, which has been applied in phase I and II of a clinical trial for cancer patients (Hu-Lowe et al., 2011; Necchi et al., 2014). BMP9-treated EBs resulted in the highest yield of angiogenic sprouting, which was robustly reduced using the anti-human ALK1 antibody (Figure 2A). hESC-ECs were harvested at day 10 and lysed for western blot analysis. SMAD1/5 activation was present in BMP9-treated hESC-ECs but absent in anti-human ALK1 antibody-treated cells (Figure 2B). Based on our observations, we overexpressed constitutively active (ca) or dominant-negative (dn) forms of ALK1 in hESC-derived mesodermal cells (day 3) under VEGF conditions for a further 6 days (Figure 2D). Then, cells were seeded on Matrigel, and tube-like formation was assessed. Only caALK1 overexpressing cells formed prominent tube-like structures (Figure 2C), which further supported the role for BMP9/ALK1-induced expansion and sprouting of hESC-ECs.

EGFL7 Is a Direct Target Gene of BMP9 and Regulates NOTCH and ERK Signaling

Proliferating ECs are enriched in ALK1 and EGFL7 expression (Parker et al., 2004; Suzuki et al., 2010). Yet, these two endothelial-specific proteins have not been linked directly. We hypothesized that EGFL7 is a downstream

(E) EBs were stimulated for mesodermal and vascular differentiation, dissociated after 10 days, and CD31⁺ cells were isolated using Dynabeads. CD31⁺ cells were seeded on Matrigel (growth factor reduced) and treated with either BMP9 or SB-431542 for 12 h. After fixation, cells were stained for VE-Cadherin and pSMAD2/3 (first row), Endoglin and pSMAD1/5 (second row), and α SMA and ID1 (last row). Scale bar, 100 μ m.

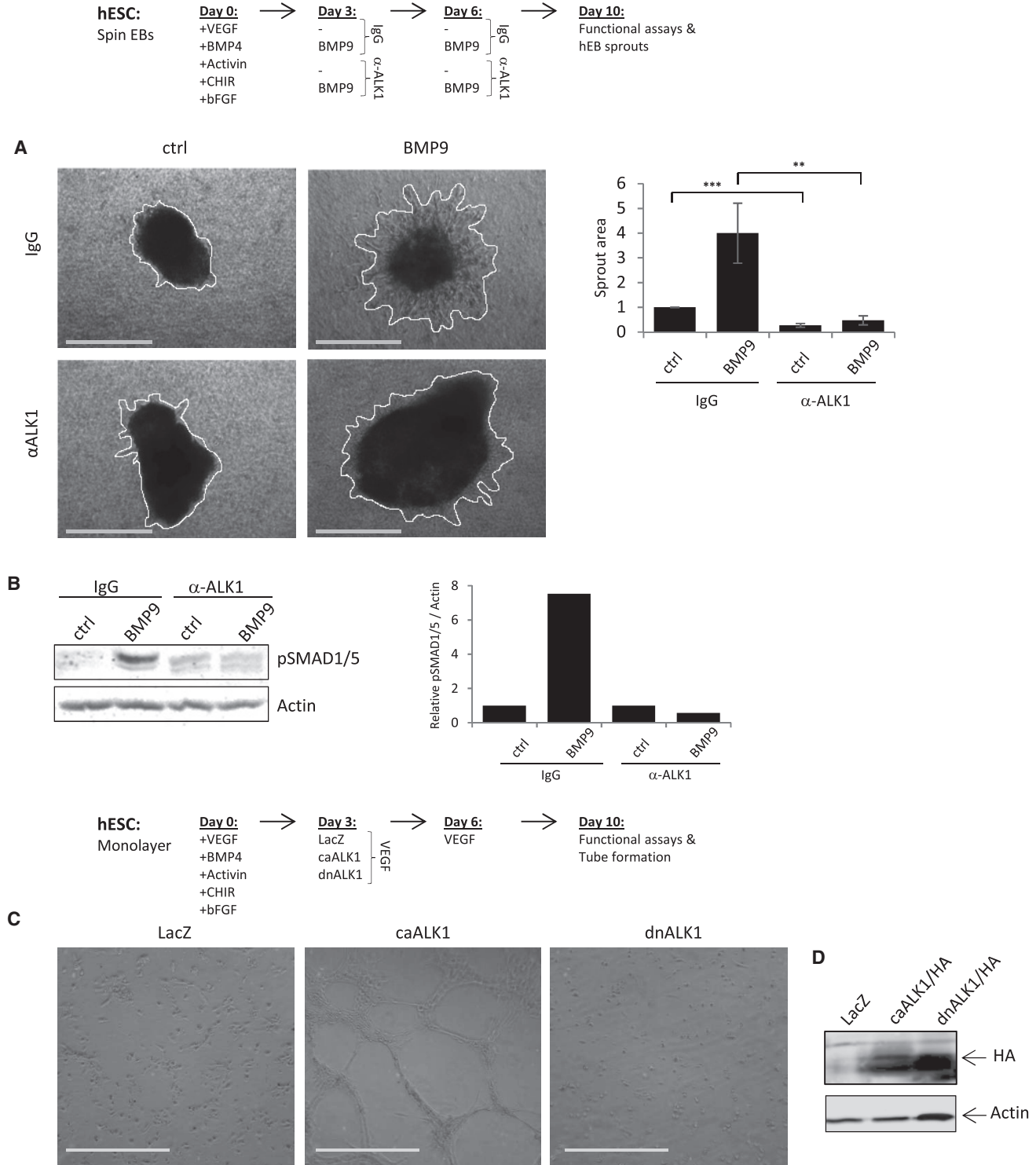


Figure 2. BMP9 Induces hESC-Derived Vascular Expansion and Endothelial Sprouting via ALK1
 (A) EBs were embedded in collagen and treated with either IgG (control) or the anti-human ALK1 neutralizing antibody PF-03446962 (Pfizer). Lasso highlights angiogenic sprouting area. Scale bar, 50 μ m. Sprout area quantified on the right (ImageJ) from three different experiments. The error bars represent SD. **p < 0.01, ***p < 0.001.

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target of the BMP9/ALK1 axis in hESC-ECs. This was substantiated by SMAD1/5 ChIP-seq data in human umbilical vein endothelial cells (HUVECs) treated with BMP9 for 90 min (Morikawa et al., 2011, #2920), which showed five SMAD1/5 binding sites in the *EGFL7* gene locus, compatible with *EGFL7* being a direct target of BMP9-SMAD1/5 signaling (Figure 3A). Moreover, *EGFL7* expression was induced upon BMP9 treatment of hESC-ECs compared with the VEGF control condition (Figure 3B). Accordingly, overexpression of caALK1 resulted in the upregulation of *EGFL7* (Figures 3C and 3D). The adenovirally infected ALK constructs were verified with respect to SMAD1/5 and SMAD2/3 phosphorylation and the expression of the downstream targets ID1, SMAD7, and PAI-1 (Figures S1C–S1E).

To understand the function of *EGFL7* in human vascular sprouting, the CRISPR-Cas9 technique was used to knock out *EGFL7*. Three different CRISPR guide RNAs (gRNAs) that bind to three different exons within the *EGFL7* gene were used to create a homozygous knockout in HUVECs (Figure S2A). A functional *EGFL7* knockout in hESCs was not achieved possibly because of an essential role of *EGFL7*. High cleavage activity (28%) was obtained in the T7EI assay for both gRNA1 and gRNA3, whereas gRNA2 exhibited no cleavage activity in HUVECs (Figure S2B). A homozygous 1 bp deletion (Figure 3E), precisely where Cas9 was expected to cleave the dsDNA, was generated in exon 6, which resulted in a frameshift and therefore in a knockout of *EGFL7* (*EGFL7*^{-/-}) using gRNA1. The lack of *EGFL7* was confirmed by western blot using an *EGFL7* antibody recognizing the whole protein (Figure 3F). The expression levels of both miR-126-5p and miR-126-3p, which are encoded in the intronic 7 sequence of *EGFL7*, were not affected in the *EGFL7*^{-/-} HUVECs (Figure S2C). In addition, *EGFL7*^{-/-} cells showed comparable levels of the endothelial marker CD31 as the wild-type (WT) HUVECs (Figures S2D and S2E). The proliferation rate was slower in *EGFL7*^{-/-} cells (data not shown).

Blood vessel formation is orchestrated by cross-talk between the BMP, NOTCH, and VEGF signaling pathways. Ectopic *EGFL7* can interact with the extracellular domains of NOTCH1–4 and thus functions as an antagonist of NOTCH activation that downregulates expression of NOTCH target genes (Schmidt et al., 2009). Ectopic *EGFL7*-NOTCH interactions result in a hyper-angiogenic response in mice (Nichol et al., 2010). Expression analysis

of *HES1* and *HEY1* in HUVECs showed significant upregulation of these genes in *EGFL7*^{-/-} cells (Figure 3G), which is consistent with the above-mentioned studies (Nichol et al., 2010; Schmidt et al., 2009). Moreover, *HES1* and *HEY1* expression in *EGFL7*^{+/-} cells was half that observed in *EGFL7*^{-/-} cells (Figure S2F). Along the same lines, BMP9 downregulated *HES1* and *HEY1* in hESC-ECs (Figure 3H), suggesting *EGFL7* as a bridge between BMP9 and NOTCH signaling regulation.

In our study, the phosphorylation status of ERK1/2 was used as a readout for activation of the MAP kinase pathway. We found that *EGFL7*^{-/-} cells exhibit attenuated ERK1/2 phosphorylation compared with WT HUVECs (Figure 3I). Interestingly, the negative regulators of the VEGF-induced MAPK pathway and phosphatidylinositol 3-kinase, *SPRED1*, and *PIK3R2*, respectively (Fish et al., 2008), were significantly upregulated in *EGFL7*^{-/-} HUVECs (Figure 3J), suggesting a regulatory role for *EGFL7* in enhancing VEGF/ERK signaling.

Assessment of mRNA and protein levels by immunostaining analysis and western blotting revealed decreased expression of the ALK1/SMAD1/5 targets *ID1* and *TMEM100* in *EGFL7*^{-/-} (Figures S2E and S3A–S3C), which could be explained by a positive feedforward loop of the BMP9-induced *EGFL7* (Itoh et al., 2004).

Taken together, *EGFL7* expression is dependent on BMP9/ALK1 signaling and has inhibitory effects on the NOTCH pathway but positive effects on the VEGF/ERK pathway, resulting in the endothelial sprouting phenotype.

BMP9-Induced *EGFL7* Regulates Extracellular Remodeling and Is Necessary for Tube Formation

To further decipher the importance of *EGFL7* in BMP9-treated hESC-ECs, short hairpin *EGFL7* (sh*EGFL7*) was lentivirally infected in hESC-derived mesodermal cells (day 3) and EC differentiation was induced in a monolayer culture for 7 days (Figure 4A). Then, cells were seeded on Matrigel and tube-like formation was assessed after 24 h. BMP9 enhanced tube formation in shControl compared with untreated cells, whereas BMP9 was unable to do so in *EGFL7* knockdown cells (Figures 4A and 4B).

Angiogenesis is regulated by dynamic interaction between ECs and the extracellular matrix (ECM) (ten Dijke and Arthur, 2007; Goumans et al., 2017). *EGFL7* is a secreted protein and is tightly associated with the

(B) Western blot of protein lysate from BMP9-induced EBs treated with hALK1 ab or IgG as a control, representing three different experiments.

(C) hESC-derived mesodermal cells were adenovirally infected with different ALK receptor constructs and then induced with VEGF toward vascular commitment. Cells were seeded on Matrigel and tube-like formation assessed (n = 3). ca; constitutively active, dn; dominant-negative. Scale bar, 100 μm.

(D) Western blot of protein lysate from the adenovirally infected cells used in (C). The ALK1 constructs are HA tagged. LacZ, control.

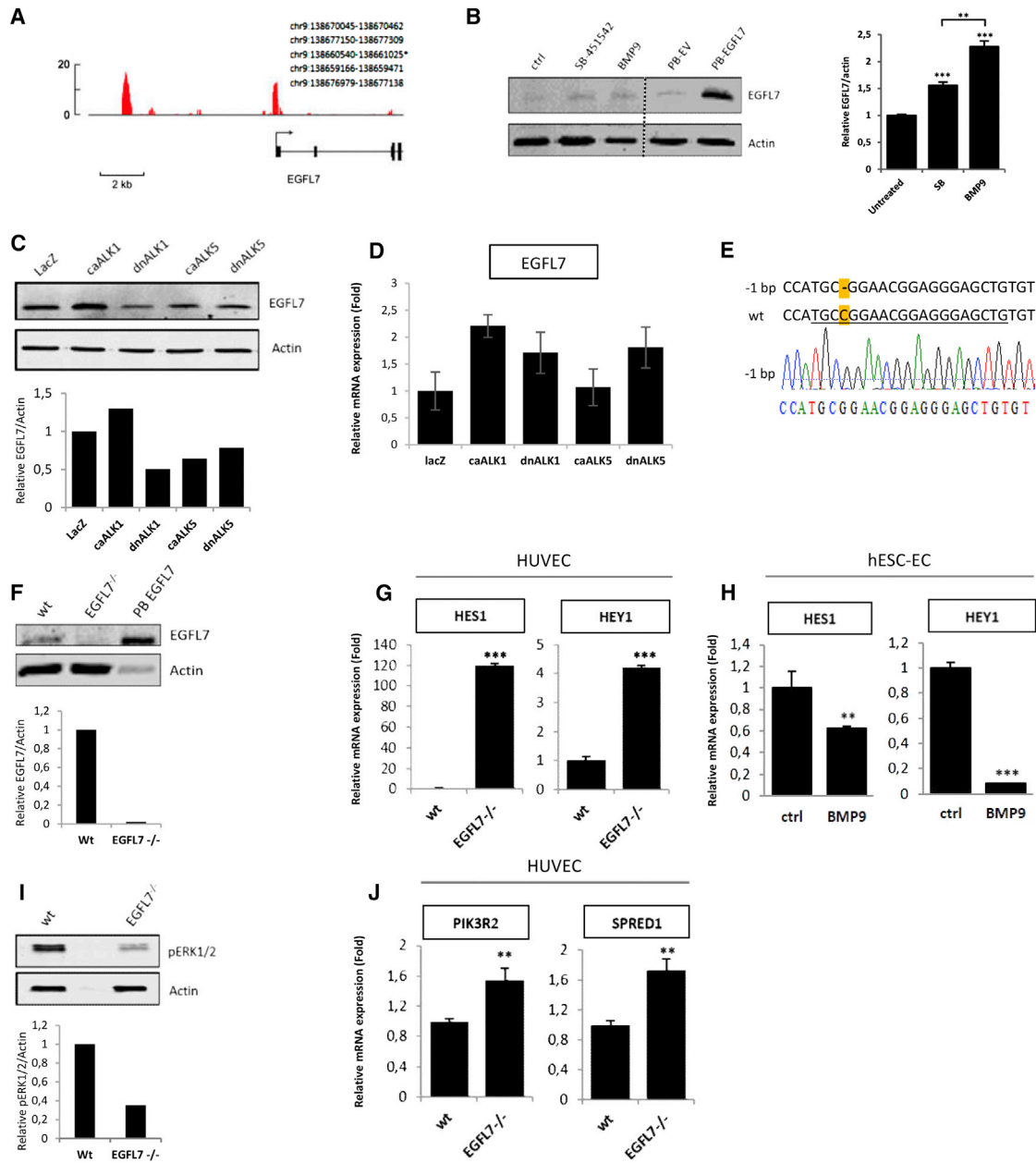


Figure 3. The BMP9/ALK1/SMAD1/5 Pathway Directly Binds to and Induces EGFL7 Expression, which Regulates NOTCH and ERK Signaling

(A) Visualization of the *EGFL7* gene locus and the results of SMAD1/5 ChIP-seq in HUVECs. Red peaks represent SMAD1/5 binding sites. The asterisk depicts the strongest binding site.

(B) Western blot of EGFL7 after treatment of hESC-ECs with BMP9 or SB-431542. EGFL7 expression was normalized to actin (right panel). PB-EGFL7, PiggyBac-EGFL7 overexpression used as a positive control. Quantification of western blot bands from three independent experiments on the right (ImageJ). The error bars represent SD. ** $p < 0.01$, *** $p < 0.001$ using unpaired Student's t test.

(C) Western blot of protein lysate from the ALK adenovirally infected hESC-ECs. EGFL7 expression and actin as a loading control.

(D) qRT-PCR of *EGFL7* after adenoviral infection of ALK constructs in hESC-ECs (normalized to human *ARP* using $\Delta\Delta$ -Ct) from three different experiments. The error bars represent SD.

(E) 1 bp deletion was generated in both alleles of the *EGFL7* gene using CRISPR and gRNA1 in HUVECs. The 1 bp deletion is highlighted with an orange box; the sgRNA1 binding site is underlined.

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interstitial ECM. Moreover, fibronectin (FN) facilitates EGFL7 deposition (Schmidt et al., 2007). We investigated the FN expression in conditions of EGFL7 deficiency and BMP9 induction. The expression of FN was significantly reduced in EGFL7^{-/-} HUVECs at both mRNA and protein levels as detected by qPCR, western blotting, and immunostaining analyses (Figures 4C–4E). FN was significantly upregulated in BMP9-treated hESC-ECs (Figure 4F).

Taken together, EGFL7 is necessary for BMP9-induced tube-like formation of hESC-ECs. FN expression is dependent on EGFL7. Whether EGFL7 regulates FN at the post-transcriptional level or as a secreted form is unknown.

DISCUSSION

We demonstrate that the BMP9/ALK1 cascade promotes sprouting of hESC-ECs. Moreover, we found evidence that the extracellular protein EGFL7 is a target gene of BMP9-induced SMAD1/5 and a key player in fine-tuning the pro- and anti-angiogenic balance by mediating inhibition of the NOTCH pathway and enhancing VEGF/ERK signaling. EGFL7 is also involved in modulating expression of ECM proteins as shown by the importance of EGFL7 for FN expression (Figure S3D).

Here, we demonstrate that ID1 is induced in EBs treated with the ALK4/5/7 inhibitor, SB-431542, but the cells expressing ID1 are incapable of invading the collagen matrix in contrast to the BMP9-induced ID1 expression cells, which are highly invasive. We suggest that this property is acquired through the active involvement of EGFL7.

In the postnatal retina, EGFL7 antagonizes NOTCH signaling, which results in a hyper-angiogenic response (Nichol et al., 2010). Our study showed an upregulation of NOTCH target genes in EGFL7^{-/-} HUVECs, indicating that BMP9-induced expression of EGFL7 could mediate the angiogenic effect by inhibiting NOTCH signaling. This is in contrast to several publications where BMP9 has been shown to upregulate NOTCH target genes. However, BMP signaling synergizes with NOTCH and antagonizes NOTCH signaling at later time points, and our cells are exposed to a long-term BMP9 treatment. Hence, EGFL7 may be the candidate in fine-tuning this cross-talk.

Several studies showing the anti-angiogenic effect of BMP9 in association with the upregulation of NOTCH target genes used a high dose of BMP9 (10 ng/mL) (David et al., 2007; Larrivee et al., 2012), whereas in this study we use 1 ng/mL BMP9. TGF- β has a biphasic effect on ECs, being pro-angiogenic at low dose (via SMAD1/5) and anti-angiogenic at high dose (via SMAD2/3) (Goumans et al., 2002). A biphasic effect could also apply to BMP9, but additional work is needed to unravel BMP9 dose dependency on NOTCH signaling and its effect on blood vessel formation.

In addition, secreted EGFL7 is involved in overall ECM composition and remodeling by direct binding of other ECM proteins in ECs (Schmidt et al., 2007). Here, we demonstrate a dramatic decrease in the expression of FN in EGFL7^{-/-} HUVECs and an induction of FN expression upon BMP9 treatment. EGFL7 can bind to integrins in ECs (Nikolic et al., 2013), but additional work is needed to study in detail the involvement of BMP9-induced EGFL7 in ECM interactions.

Defects in BMP signaling in the vascular bed result in diseases such as HHT and PAH. Elucidating the signaling cascades that have instructive roles in vasculogenesis and angiogenesis is therefore of great importance. Moreover, improving vascular development in culture can feedforward the use of organoids as a source for large engineered tissues.

EXPERIMENTAL PROCEDURE

Cell Culture of hESCs and HUVECs

The Icelandic Bioethics committee has approved the application of hESC lines for our research (permit 05-079). hESC lines HES2 and H1 (WiCell Research Institute, Madison, WI) were obtained as trypsin-adapted variants from the Keller laboratory. hESC lines were routinely cultured on a monolayer of mitomycin C-treated primary mouse embryonic fibroblasts (MEFs CF-1 strain, CellSystems). Medium consisted of 3/4 DMEM/F12 (Invitrogen), supplemented with 20% KnockOut Serum Replacement (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 200 nM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen),

(F) Western blot of EGFL7 and actin expression from protein samples of WT and EGFL7^{-/-} HUVECs. PB-EGFL7 overexpression used as a positive control.

(G and H) qRT-PCR of the NOTCH target genes (*HES1*, *HEY1*) (normalized to human *ARP* using $\Delta\Delta^{-Ct}$) in WT and EGFL7^{-/-} HUVECs (G) and untreated versus BMP9-treated hESC-ECs (H). Graphs represent one of three independent experiments. The error bars represent SD. **p < 0.01, ***p < 0.001 using unpaired Student's t test.

(I) Western blot of phosphorylated ERK1/2 and actin expression (loading control) from protein samples of WT and EGFL7^{-/-} HUVECs.

(J) qRT-PCR of the negative regulators of the VEGF-induced MAPK pathway and phosphatidylinositol 3-kinase *SPRED1* and *PIK3R2* (normalized to human *ARP* using $\Delta\Delta^{-Ct}$) in WT and EGFL7^{-/-} HUVECs (n = 3). The error bars represent SD. **p < 0.01 using unpaired Student's t test.

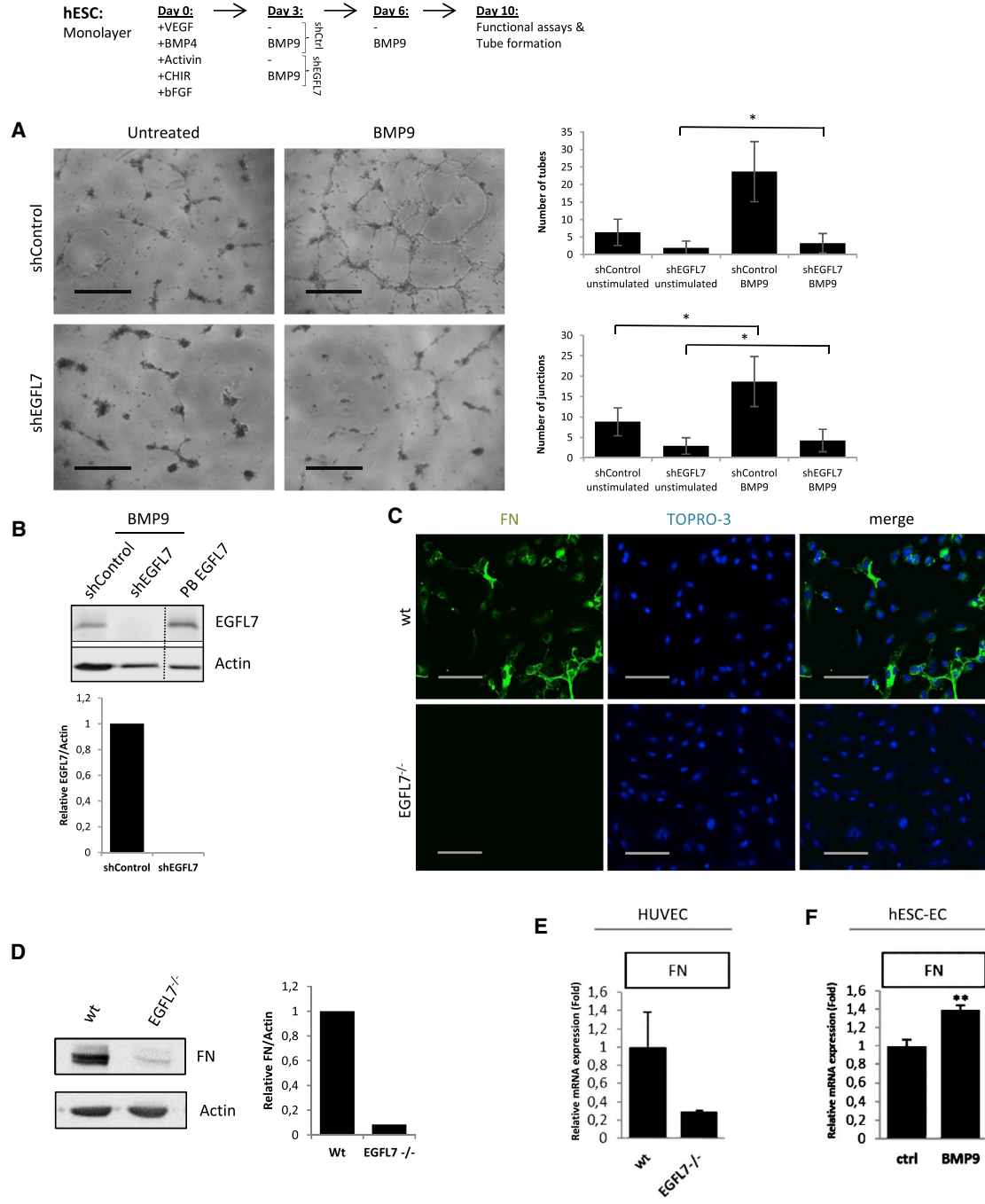


Figure 4. EGFL7 Affects Extracellular Matrix Proteins

(A) hESC-derived mesodermal cells were lentivirally infected with shEGFL7 or shControl constructs in pLKO.1 plasmid and selected on puromycin and induced toward vascular cells according to protocol. Two shRNA-EGFL7 were combined for infection. Cells were seeded on Matrigel and tube-like formation assessed. sh, short hairpin. Scale bar, 100 μm. Number of tubes and junctions were quantified (ImageJ) from three independent experiments. The error bars represent SD. *p < 0.05.

(B) Western blot of EGFL7 knockdown. EGFL7 expression and actin (used as a loading control). PB-EGFL7, PiggyBac-EGFL7 overexpression as a positive control.

(C) Immunofluorescent staining of WT and EGFL7^{-/-} HUVECs against fibronectin (FN) and TOPRO-3 (blue) nuclear staining. Scale bar, 100 μm.

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55 mM 2-mercaptoethanol (Invitrogen) mixed with 1/4 mTesR1 (STEMCELL Technologies). For mesodermal and vascular differentiation, hESCs were cultured in BPEL medium (Ng et al., 2008) feeder free on Matrigel-coated (BD) (diluted 1:100 in DMEM/F12 without supplements) culture dishes. TrypLE (Invitrogen) was used to dissociate the cells for splitting and expansion. The HUVECs were cultured in EGM-2 medium (Lonza) on 0.1% gelatin-coated cultures flasks.

Statistical Analysis

Experiments were conducted in triplicate and data are presented as means \pm SD. Significance of differences was measured by an unpaired Student's t test. The following p values were considered statistically significant: *p < 0.05, **p < 0.01, ***p < 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.04.022>.

AUTHOR CONTRIBUTIONS

G.V. conceived and designed the project. A.R., M.S.A., S.H.M., and G.V. performed the experiments. T.R.R. designed the gRNA-CRISPR and performed the T7EI analysis and M.M. analyzed the ChIP-seq data. A.R., A.Z., and G.V. wrote the manuscript.

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(D) Western blot of FN and actin expression from protein samples of WT and EGFL7^{-/-} HUVECs.

(E and F) qRT-PCR of FN. (E) WT and EGFL7^{-/-} HUVECs (F) untreated versus BMP9-treated hESC-ECs. All values were normalized to human *ARP* using $\Delta\Delta^{-Ct}$. Graphs represent one of three experiments. The error bars represent SD. **p < 0.01 using unpaired Student's t test.



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