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

Anne Richter,1 Marta S. Alexdottir,2 Svala H. Magnus,1 Tobias R. Richter,2 Masato Morikawa,3 An Zwijsen,4,5 and Gudrun Valdimarsdottir1,2,*
1Department of Biochemistry and Molecular Biology, BioMedical Center, University of Iceland, Vatnsmyrarvegur 16, 101 Reykjavik, Iceland
2Department of Anatomy, BioMedical Center, University of Iceland, Sturlugata 8, 101 Reykjavik, Iceland
3Ludwig Institute for Cancer Research, Uppsala University, 751 24 Uppsala, Sweden
4VIB-KU Leuven Center for Brain and Disease Research, ON4 Herestraat 49, Box 602, 3000 Leuven, Belgium
5KU Leuven Department of Cardiovascular Sciences, ON4 Herestraat 49, Box 911, 3000 Leuven, Belgium
*Correspondence: gudrunva@hi.is
https://doi.org/10.1016/j.stemcr.2019.04.022

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 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 μm.
(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 μm.
(legend continued on next page)
on ECs. Expression of EGFL7 is highest in proliferating vasculature and regulates tubulogenesis in zebrafish and vascular patterning and integrity in mice (Nicol 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 down-regulation 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 aSMA 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
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 μm. Sprout area quantified on the right (ImageJ) from three different experiments. The error bars represent SD. **p < 0.01, ***p < 0.001.

(legend continued on next page)
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 cALK1 resulted in the up-regulation 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\textsuperscript{−/−}) 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 the EGFL7 gene locus, were not affected in the EGFL7\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsuperscript{−/−} cells was half that observed in EGFL7\textsuperscript{+/−} 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\textsuperscript{−/−} 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, SPTD1, and PIK3R2, respectively (Fish et al., 2008), were significantly upregulated in EGFL7\textsuperscript{−/−} 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\textsuperscript{−/−} (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 (shEGFL7) was lentivirally infected in hESC-ECs (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...
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 ∆∆ 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.

(legend continued on next page)
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 up-regulated 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 feed-forward 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), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% non-essential amino acids (Invitrogen), 1% n...
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.

(legend continued on next page)
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 ± 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.

**ACKNOWLEDGMENTS**

We are grateful to Dr. H. Halldorsson for HUVECs and Dr. ten Dijke for providing the shEGFL7 construct and constructive discussions. We appreciate helpful discussions with Dr. M.K. Magnusson and Dr. E. Steingrimsson. We thank Dr. C. Mummery and Dr. P. Knaus for valuable suggestions on this work and T. Wachsmann and Dr. S. Ingthorsson for expert technical assistance. This research was supported by the Recruitment Fund of the University of Iceland (grant no. HI14080107) and a project grant (grant no. 110435021) supported by the Icelandic Centre for Research (RANNIS).

Received: July 24, 2018
Revised: April 26, 2019
Accepted: April 30, 2019
Published: May 30, 2019

**REFERENCES**

David, L., Mallet, C., Mazebourg, S., Feige, J.J., and Bailly, S. (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109, 1953–1961.

Fish, J.E., Santoro, M.M., Morton, S.U., Yu, S., Yeh, R.F., Wythe, J.D., Ivey, K.N., Bruneau, B.G., Stainier, D.Y., and Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. Dev. Cell 15, 272–284.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. 21, 1743–1753.

Goumans, M.J., Zwijsen, A., Ten Dijke, P., and Bailly, S. (2017). Bone morphogenetic proteins in vascular homeostasis and disease. Cold Spring Harb Perspect. Biol. 10. https://doi.org/10.1101/cshperspect.a031989.

Hollnegel, A., Oehlmann, V., Heymer, J., Ruther, U., and Nordheim, A. (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J. Biol. Chem. 274, 19838–19845.

Hu-Lowe, D.D., Chen, E., Zhang, L., Watson, K.D., Mancuso, P., Lappin, P., Wickman, G., Chen, J.H., Wang, J., Jiang, X., et al. (2011). Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. Cancer Res. 71, 1362–1373.

Itoh, F., Itoh, S., Goumans, M.J., Valdimarsdottir, G., Iso, T., Dotto, G.P., Hamamori, Y., Kedes, L., Kato, M., and ten Dijke Pt, P. (2004). Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. 23, 541–551.

James, D., Nam, H.S., Seandel, M., Nolan, D., Janovitz, T., Tomishima, M., Studer, L., Lee, G., Lyden, D., Benezra, R., et al. (2010). Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. Nat. Biotechnol. 28, 161–166.

Larrivee, B., Praehst, C., Gordon, E., del Toro, R., Mathivet, T., Duarte, A., Simons, M., and Eichmann, A. (2012). ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev. Cell 22, 489–500.

Lebrin, F., Srun, S., Raymond, K., Martin, S., van den Brink, S., Freitas, C., Breant, C., Mathivet, T., Larrivee, B., Thomas, J.L., et al. (2010). Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. Nat. Med. 16, 420–428.

Lyden, D., Young, A.Z., Zagzag, D., Yan, W., Gerald, W., O’Reilly, R., Bader, B.L., Hynes, R.O., Zhuang, Y., Manova, K., et al. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature 401, 670–677.

Morikawa, M., Koinuma, D., Tsutsumi, S., Vasilaki, E., Kanki, Y., Heldin, C.H., Aburatani, H., and Miyazono, K. (2011). ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a binding motif. Nucleic Acids Res. 39, 8712–8727.

Morrell, N.W., Bloch, D.B., ten Dijke, P., Goumans, M.J., Hata, A., Smith, J., Yu, P.B., and Bloch, K.D. (2016). Targeting BMP signalling...
in cardiovascular disease and anaemia. Nat. Rev. Cardiol. 13, 106–120.
Moya, I.M., Umans, L., Maas, E., Pereira, P.N., Beets, K., Francis, A., Sents, W., Robertson, E.J., Mummery, C.L., Huylebroeck, D., et al. (2012). Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades. Dev. Cell 22, 501–514.
Necchi, A., Giannatempo, P., Mariani, L., Fare, E., Raggi, D., Penny, M., Zaffirini, N., Crippa, F., Marchiano, A., Nicolai, N., et al. (2014). PF-03446962, a fully-human monoclonal antibody against transforming growth-factor beta (TGFbeta) receptor ALK1, in pre-treated patients with urothelial cancer: an open label, single-group, phase 2 trial. Invest. New Drugs 32, 555–560.
Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protoc. 3, 768–776.
Nichol, D., Shawber, C., Fitch, M.J., Bambino, K., Sharma, A., Kitajewski, J., and Stuhlmann, H. (2010). Impaired angiogenesis and altered Notch signaling in mice overexpressing endothelial Egfl7. Blood 116, 6133–6143.
Nichol, D., and Stuhlmann, H. (2012). EGFL7: a unique angiogenic signaling factor in vascular development and disease. Blood 119, 1345–1352.
Nikolic, I., Stankovic, N.D., Bicker, F., Meister, J., Braun, H., Awwad, K., Baumgart, J., Simon, K., Thal, S.C., Patra, C., et al. (2013). EGFL7 ligates alphavbeta3 integrin to enhance vessel formation. Blood 121, 3041–3050.
Parker, L.H., Schmidt, M., Jin, S.W., Gray, A.M., Beis, D., Pham, T., Frantz, G., Palmieri, S., Hillan, K., Stainier, D.Y., et al. (2004). The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. Nature 428, 754–758.
Poon, E., Clermont, F., Firpo, M.T., and Akhurst, R.J. (2006). TGFbeta inhibition of yolk-sac-like differentiation of human embryonic stem-cell-derived embryoid bodies illustrates differences between early mouse and human development. J. Cell Sci. 119, 759–768.
Richter, A., Valdimarsdottir, L., Hafnkelsdottir, H.E., Runnarsson, J.E., Omarsdottir, A.R., Ward-van Oostwaard, D., Mummery, C., and Valdimarsdottir, G. (2014). BMP4 promotes EMT and mesodermal commitment in human embryonic stem cells via Slug and MSX2. Stem Cells 32, 636–648.
Schmidt, M., Paes, K., De Maziere, A., Snyman, T., Yang, S., Gray, A., French, D., Kasman, I., Klumperman, J., Rice, D.S., et al. (2007). EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. Development 134, 2913–2923.
Schmidt, M.H., Bicker, F., Nikolic, I., Meister, J., Babuke, T., Picuric, S., Muller-Esterl, W., Plate, K.H., and Dikic, I. (2009). Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. Nat. Cell Biol. 11, 873–880.
Suzuki, Y., Ohga, N., Morishita, Y., Hida, K., Miyazono, K., and Wattage, T. (2010). BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. J. Cell Sci. 123, 1684–1692.
ten Dijke, P., and Arthur, H.M. (2007). Extracellular control of TGFbeta signalling in vascular development and disease. Nat. Rev. Mol. Cell Biol. 8, 857–869.
Valdimarsdottir, G., Goumans, M.J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P., and ten Dijke, P. (2002). Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 106, 2263–2270.
Vallier, L., Alexander, M., and Pedersen, R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. 118, 4495–4509.
Wang, S., Aurora, A.B., Johnson, B.A., Qi, X., McAnally, J., Hill, J.A., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev. Cell 15, 261–271.