

Homeobox D3, A Novel Link Between Bone Morphogenetic Protein 9 and Transforming Growth Factor Beta 1 Signaling

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<https://doi.org/10.1016/j.jmb.2020.01.043>

Edited by M Yaniv

Abstract

Aims

Several signaling pathways contribute to endothelial-mesenchymal transitions and vascular calcification, including bone morphogenetic protein (BMP) and transforming growth factor (TGF) β signaling. The transcription factor homeobox D3 (*Hoxd3*) is known to regulate an invasive endothelial phenotype, and the aim of the study is to determine if HOXD3 modulates BMP and TGF β signaling in the endothelium.

Methods and Research

We report that the endothelium with high BMP activity due to the loss of BMP inhibitor matrix Gla protein (MGP) shows induction of *Hoxd3*. HOXD3 is part of a BMP-triggered cascade. When activated by BMP9, activin receptor-like kinase (ALK) 1 induces HOXD3 expression. *Hoxd3* promoter is a direct target of phosphorylated (p) SMAD1, a mediator of BMP signaling. High BMP activity further results in enhanced TGF β signaling due to induction of TGF β 1 and its receptor, ALK5. This is mediated by HOXD3, which directly targets the *Tgfb1* promoter. Finally, TGF β 1 and BMP9 stimulate the expression of MGP, which limits the enhanced ALK1 induction by counteracting BMP4. The cascade of BMP9-HOXD3-TGF β also affects Notch signaling and angiogenesis through induction of Notch ligand Jagged 2 and suppression of Notch ligand delta-like 4 (Dll4).

Conclusion

The results suggest that HOXD3 is a novel link between BMP9/ALK1 and TGF β 1/ALK5 signaling.

Translational perspective

BMP and TGF β signaling are instrumental in vascular disease such as vascular calcification and atherosclerosis. This study demonstrated a novel type of cross talk between endothelial BMP and TGF β signaling as mediated by HOXD3. The results provide a possible therapeutic approach to control dysfunctional BMP and TGF β signaling by regulating HOXD3.

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Introduction

HOXD3 is part of the HOXD gene family and plays an important role in developmental processes, such as the formation of the somatic mesoderm and neural crest [1,2]. HOXD3 is also involved in the conversion of the endothelium from quiescence to the angiogenic state [3–5]. Interestingly, when targeted by microRNA miR-203a, HOXD3 can also suppress angiogenesis and cell metastasis in hepatocellular carcinoma through the vascular endothelial growth factor (VEGF) receptor [6]. The HOXD3 expression is associated with a positional identity in endothelial cells (ECs), exhibiting high levels of HOXD3 in venous ECs and moderate levels in arterial ECs [7].

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF β) superfamily and are critical for angiogenesis in embryos, as well as for vascular homeostasis in adults [8–10]. The BMP and TGF β ligands elicit their activity through the activation of receptor complexes composed of type I and type II Ser/Thr kinase receptors. Seven type I receptors, termed activin receptor–like kinase (ALK1 to ALK7), determine the specificity of BMP signal in concert with five type II receptors [8]. When BMP or TGF β bind the receptor complex, the type II receptor activates the type I receptor, which typically propagates the signaling by phosphorylating SMADs. BMP signaling preferentially activate receptor-activated (R)-SMAD1, 5 and 8, while TGF β signaling phosphorylates SMAD 2 and 3 [11–13]. The activity of the BMPs is modulated by a number of extracellular antagonists such as matrix Gla protein (MGP) [14], which is highly expressed in the endothelium where it primarily regulates BMP4 [15].

Here, we show that the lack of MGP increases the expression of HOXD3 through upregulation of the BMP9/ALK1 pathway. The absence of MGP allows BMP4 to induce ALK1 [16]. HOXD3 subsequently binds to the promoter of TGF β 1 and triggers its expression, resulting in regulatory effects on MGP and Notch signaling. Together, the study suggests that HOXD3 is a novel link between BMP and TGF β signaling.

Results

BMP activation induces HOXD3 in the vascular endothelium

To assess the relationship between BMP activation and HOXD3 in the vasculature, we examined the expression of HOXD3 in *Mgp*^{−/−} aortas on postnatal day (P) 1–28, when BMP signaling is highly activated [17–19]. The results showed a

significant induction of HOXD3 starting on P14 as determined by real-time polymerase chain reaction (PCR) (Fig. 1a). Similar levels of HOXD3 expression were maintained through P28 (Fig. 1a and b), when most of the *Mgp*^{−/−} mice died because of vascular abnormalities [16,20]. Immunostaining showed that HOXD3 colocalized with the endothelial marker von Willebrand factor (Fig. 1c), suggesting that the endothelium is responsible for the HOXD3 increase. We also found that the induction of HOXD3 was accompanied by enhanced expression of TGF β 1, ALK1, and ALK5, as detected by real-time PCR, immunoblotting, and immunostaining (Fig. 1d–f). This points to the possibility that BMP-induced HOXD3 is involved in TGF β signaling.

To investigate how BMP signaling induced HOXD3 expression, we performed three sets of experiments. First, we depleted MGP in human umbilical vein endothelial cells (HUVECs) by using lentiviral vectors carrying MGP siRNA. The depletion was confirmed by a reduction in MGP expression as detected by real-time PCR (Fig. 2a) and increased levels of pSMAD1, as detected by immunoblotting and immunostaining (Fig. 2b). Real-time PCR, immunoblotting, and immunostaining all showed that the depletion of MGP induced HOXD3 (Fig. 2c–e). Since previous studies showed that BMP9/ALK1 is highly induced in MGP deficiency [21], we next inhibited BMP9 activity in the MGP-depleted HUVECs by using anti-ALK1 neutralizing antibodies (10 μ g/ml). As expected, we found that suppression of BMP9/ALK1 signaling abolished the induction of HOXD3 (Fig. 3a and c), suggesting that BMP9/ALK1 signaling mediated the HOXD3 induction. Finally, we treated the HUVECs with BMP9 (10 ng/ml) for 24 h and confirmed BMP activation by immunostaining for pSMAD1 (Fig. 3b). We found that BMP9 induced the expression of HOXD3 as determined by real-time PCR, immunoblotting, and immunostaining (Fig. 3c).

To examine the pSMAD1 DNA-binding site in the promoter of the *Hoxd3* gene, we performed chromatin immunoprecipitation (ChIP) assays using anti-pSMAD1 antibodies to isolate pSMAD1-enriched genomic DNA from *Mgp*^{−/−} aortas. The results revealed a significant enhancement of pSMAD1 DNA binding in the promoter of the *Hoxd3* gene (Fig. 3d and e). Together, the results suggest that BMP9/ALK1 signaling induces HOXD3 and that *Hoxd3* is a direct target of SMAD1.

Enhanced HOXD3 induces TGF β signaling

Our results showed that BMP activation induced TGF β 1 and ALK5 in the *Mgp*^{−/−} aortas (Fig. 1). We also found induction of TGF β 1 and ALK5 in BMP9-treated HUVECs, as determined by real-time PCR, immunoblotting, and immunostaining (Fig. 4a) with a significant increase in pSMAD2/3 levels (Fig. 4b),

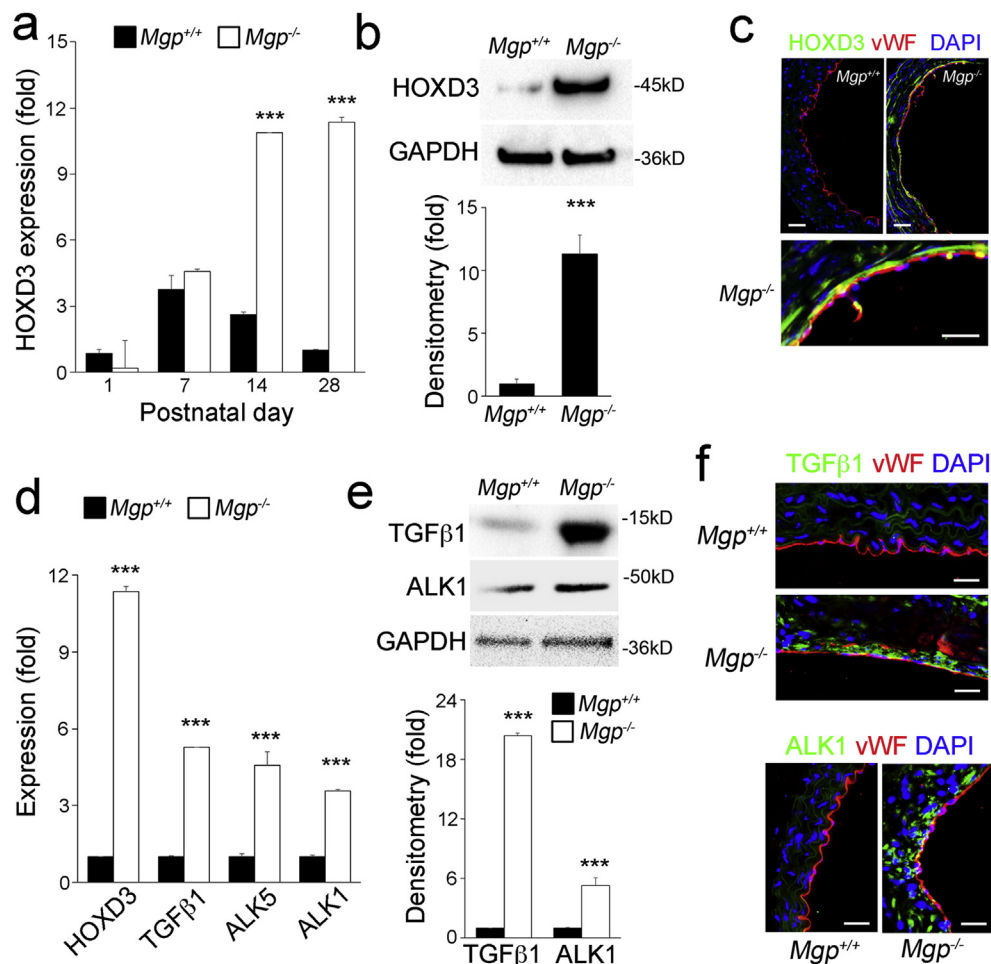


Fig. 1. Lack of MGP induces HOXD3 expression and TGF β /BMP signaling in aortic tissue. (a–c) Expression of HOXD3 in *Mgp*^{−/−} aortic tissue examined between postnatal day (P) 1–28 by real-time PCR (a), immunoblotting with densitometric analysis (b) and immunostaining on P28 (c) (n = 6). (d–e) Expression of HOXD3, TGF β 1, ALK5, ALK1 in *Mgp*^{−/−} aortic tissue was examined on P28 by real-time PCR (d) and immunoblotting with densitometric analysis (e). (f) Immunostaining of ALK1 and TGF β 1 in *Mgp*^{−/−} aortic tissue on P28. Data were analyzed by the Student's *t*-test. ***, *P* < 0.001. Error bars are standard deviation. ***, *P* < 0.001. Scale bar, 50 μ m. MGP, matrix Gla protein; HOXD3, homeobox D3; TGF β , transforming growth factor beta; BMP, bone morphogenetic protein; ALK, activin receptor–like kinase; PCR, polymerase chain reaction.

suggesting that the increased BMP9/ALK1 activity also enhanced TGF β signaling.

To investigate if HOXD3 mediated the increase in TGF β signaling, we infected the HUVECs with lentiviral vectors overexpressing HOXD3. The overexpression was confirmed by real-time PCR (Fig. 4c). The enhanced HOXD3 induced both the expression of TGF β 1 and ALK5 (Fig. 4d) and pSMAD2 signaling (Fig. 4e), suggesting that HOXD3 mediates the BMP9 effect on TGF β signaling.

To determine if TGF β 1 was a direct downstream target of HOXD3, we examined two putative HOXD3-binding sites in the promoter region of the *Tgfb1* gene using ChIP assays (Fig. 4f). The results

revealed that HOXD3 bound strongly to the two binding sites, which were located around 0.3 and 3 kb upstream of the *Tgfb1* gene (Fig. 4g). Together, our results suggested that the *Tgfb1* gene was a direct target of HOXD3.

HOXD3 induces MGP through the induction of TGF β 1, which provides negative feedback regulation for BMP4

To examine whether HOXD3 provides negative feedback for BMP9/ALK1 signaling, we treated the HUVECs with TGF β 1 (10 ng/ml) for 24 h. The increased TGF β 1 activity was confirmed by an increase in pSMAD2 immunostaining (Fig. 5a).

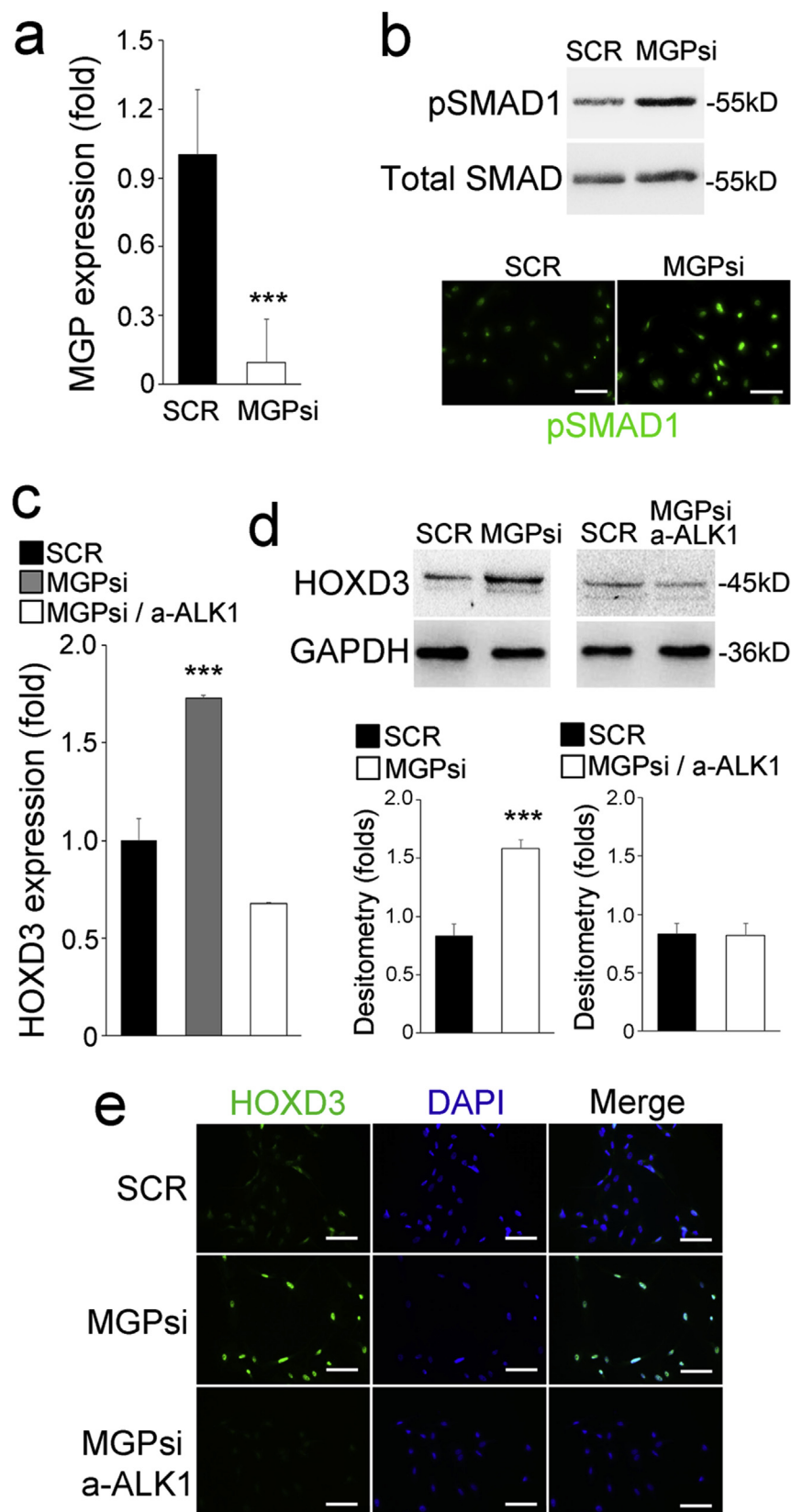


Fig. 2. Depletion of MGP increases HOXD3 expression in HUVECs. (a) Expression of MGP in HUVECs transfected with MGP siRNA. (b) Levels of pSMAD1 detected by immunoblotting and immunostaining. (c–e) HOXD3 expression in

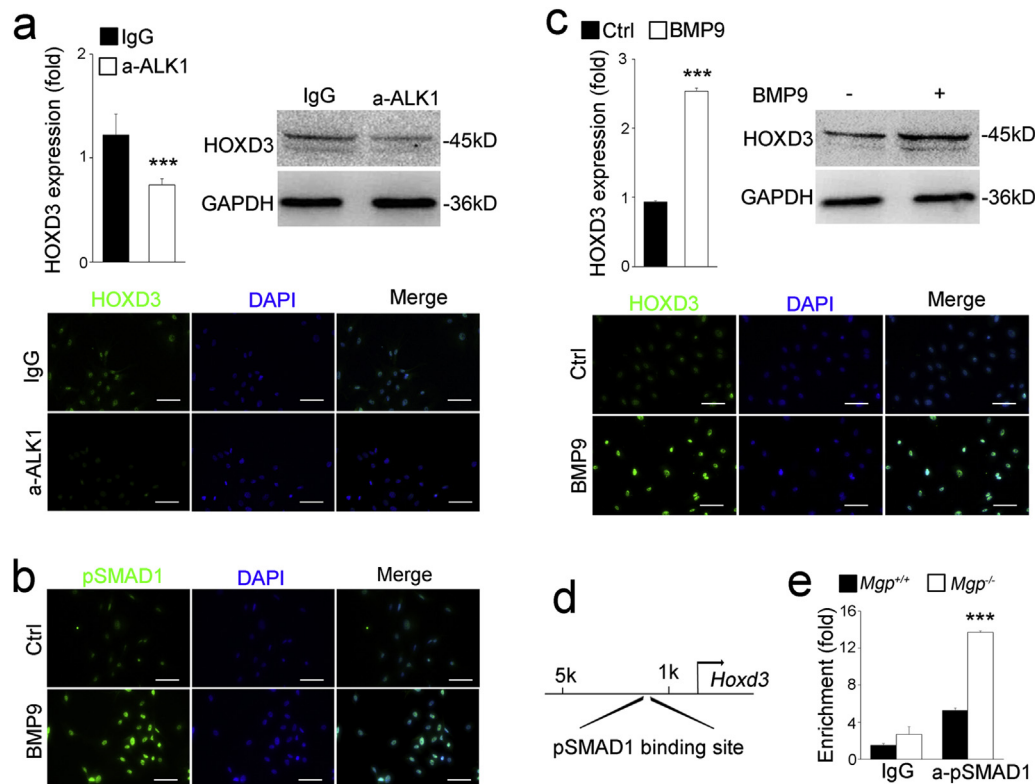


Fig. 3. BMP9 induced the expression of HOXD3 through the ALK1 and pSMAD1. (a) Expression of HOXD3 in HUVECs treated by anti-ALK1 neutralizing antibodies as shown by real-time PCR, immunoblotting, and immunofluorescence staining. (b) Level of pSMAD1 in HUVECs treated with BMP9 as shown by immunostaining. (c) Induction of HOXD3 in HUVECs treated by BMP9 as shown by real-time PCR, immunoblotting, and immunostaining. (d) pSMAD1 DNA-binding site in the promoter of the *Hoxd3* gene. (e) Increased pSMAD1 DNA binding at the *Hoxd3* promoter in *Mgp*^{-/-} aortas as shown by ChIP assays. Data were analyzed by the Student's *t*-test. ***, *P* < 0.001. Error bars are standard deviation. ***, *P* < 0.001. Scale bar, 50 μ m. MGP, matrix Gla protein; HOXD3, homeobox D3; BMP, bone morphogenetic protein; ALK, activin receptor-like kinase; HUVECs, human umbilical vein endothelial cells; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction.

The results showed that the TGF β 1 treatment reduced the expression of HOXD3 but increased MGP expression as shown by real-time PCR and immunoblotting (Fig. 5b and c). Interestingly, restoration of BMP9 signaling counteracted the effect of TGF β 1 and normalized HOXD3 expression but further enhanced MGP expression (Fig. 5b and c). Since our previous study showed that ALK1 expression was induced by BMP4 but suppressed by MGP [16], an increase in MGP would suppress ALK1 expression and provide negative feedback for BMP4 and then BMP9/ALK1 signaling (Fig. 5f) [22].

HOXD3 balances Notch signaling in angiogenesis

Previous studies have shown that BMP9/ALK1 signaling affects the expression of Notch components [23,24]. Interestingly, we found that both BMP9 treatment and HOXD3 overexpression affected Notch ligands in HUVECs. The results showed an induction of the Notch ligand Jagged 2 and a reduction of the delta-like 4 (DII4) ligand, as determined by real-time PCR (Fig. 6a and b). These effects were abolished by either anti-ALK1 neutralizing antibodies or the TGF β inhibitor SB431542, an inhibitor of ALK5 (Fig. 5c). It suggests that the cross

HUVECs transfected with MGP siRNA and treated with anti-ALK1 antibodies, as shown by real-time PCR (c), immunoblotting with densitometric analysis (d), and immunostaining (e). Data were analyzed by the Student's *t*-test. ***, *P* < 0.001. Error bars are standard deviation. ***, *P* < 0.001. Scale bar, 50 μ m. MGP, matrix Gla protein; HOXD3, homeobox D3; ALK, activin receptor-like kinase; HUVECs, human umbilical vein endothelial cells; SCR, scrambled siRNA; PCR, polymerase chain reaction.

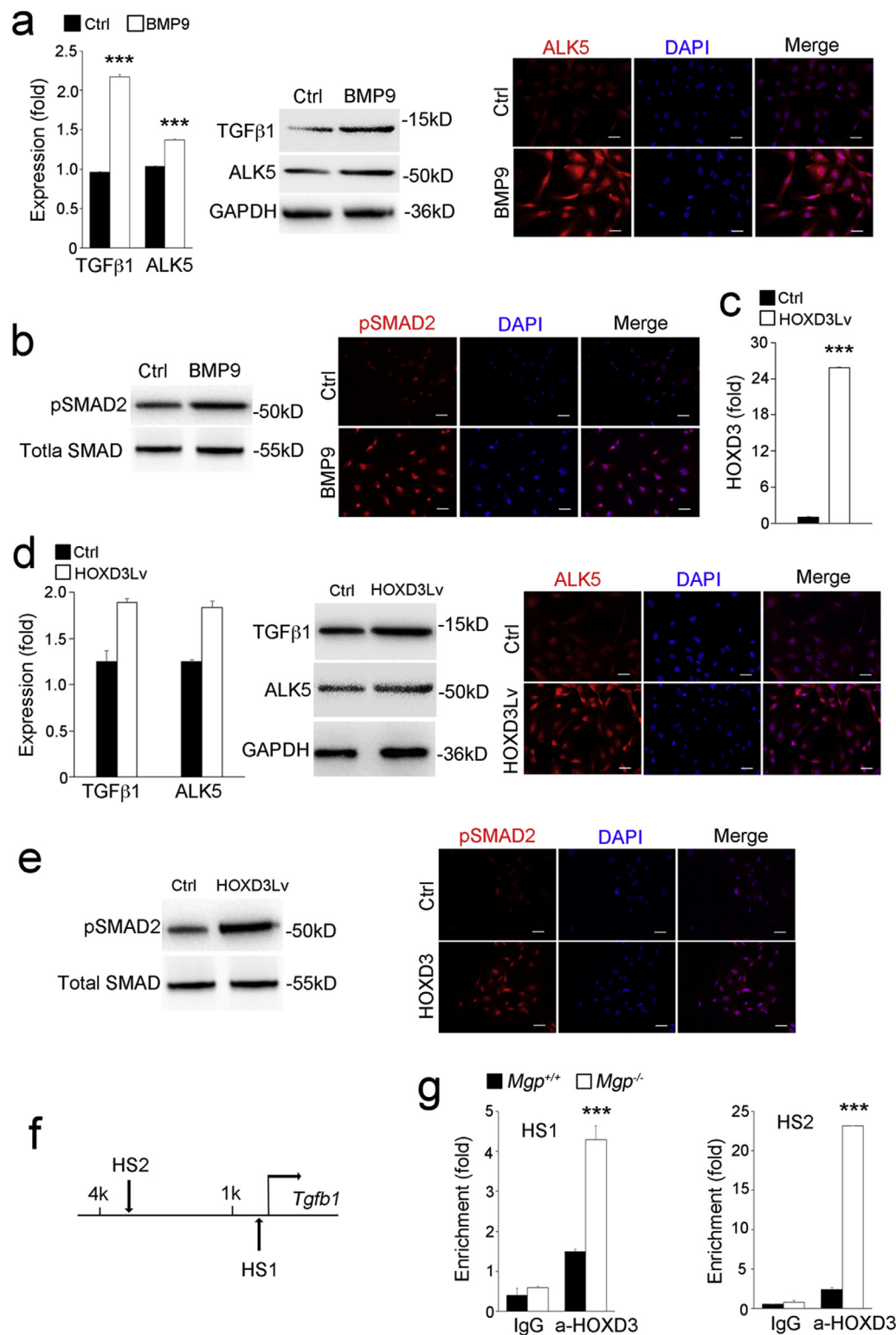


Fig. 4. BMP9 induces TGF β signaling through induction of HOXD3. (a), Expression of TGF β 1 and ALK5 in HUVECs treated with BMP9 as shown by real-time PCR, immunoblotting, and immunostaining. (b) Activation of pSMAD2 in HUVECs treated with BMP9 as shown by immunoblotting and immunostaining. (c–d) Expression of HOXD3, TGF β 1, and ALK5 in HUVECs after infection with lentiviral vectors overexpressing HOXD3, as shown by real-time PCR, immunoblotting, and immunostaining. (e) Activation of pSMAD2 in HUVECs after infection with lentiviral vectors overexpressing HOXD3, as shown by immunoblotting and immunostaining. (f) HOXD3 DNA-binding sites (HS) in the

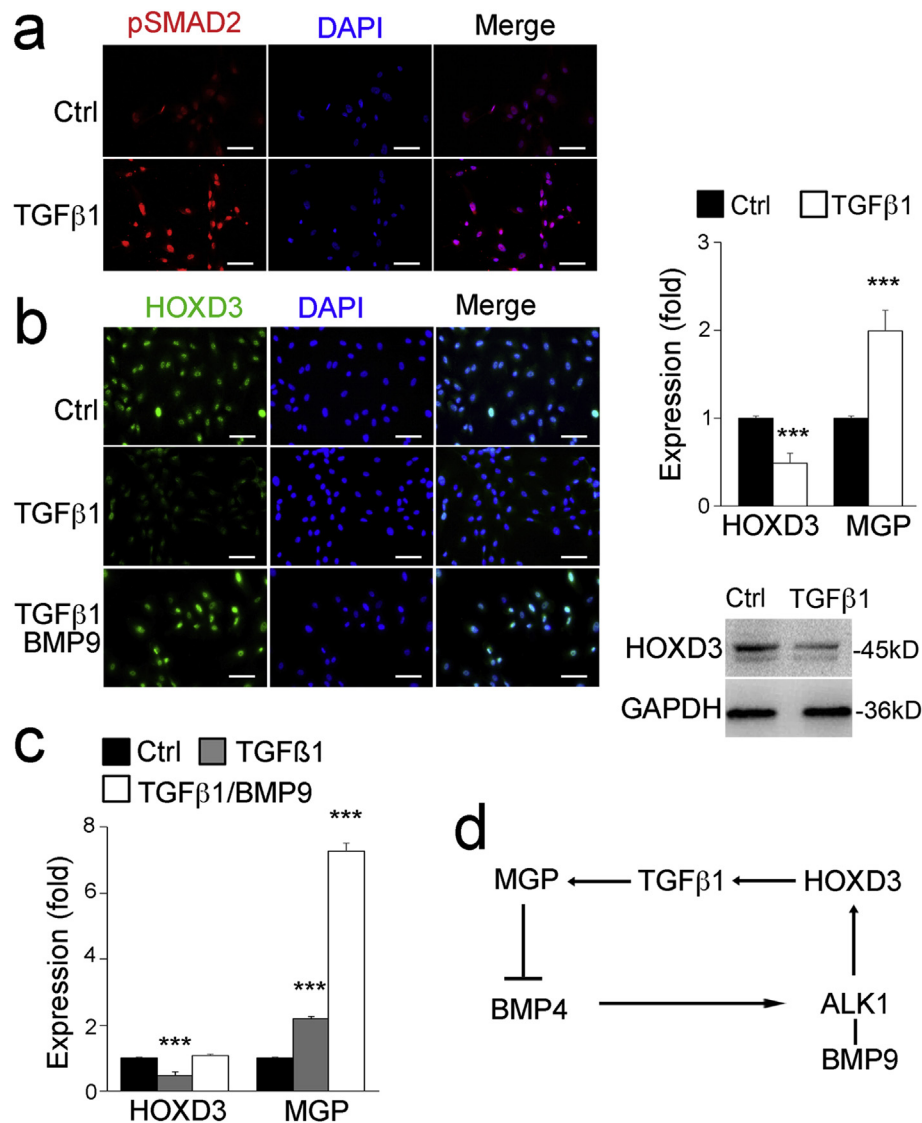


Fig. 5. TGF β 1 feedback regulates the expression of BMP9, HOXD3, and MGP. (a) The level of pSMAD2 in HUVECs after treatment with TGF β 1 as shown by immunostaining. (b–c), Expression of HOXD3 and MGP in HUVECs after treatment with TGF β 1, with or without BMP9, shown by real-time PCR, immunostaining, and immunoblotting. (d) Schematic diagram. Data were analyzed by the Student's *t*-test. ***, *P* < 0.001. Error bars are standard deviation. ***, *P* < 0.001. Scale bar, 50 μ m. MGP, matrix Gla protein; HOXD3, homeobox D3; TGF β , transforming growth factor beta; BMP, bone morphogenetic protein; HUVECs, human umbilical vein endothelial cells; PCR, polymerase chain reaction.

talk between BMP9/ALK1 and TGF β /ALK5 signaling, as mediated by HOXD3, also affects the Notch components.

To determine if HOXD3-regulated Notch signaling affects angiogenesis, we performed angiogenesis

and migration assays using HOXD3-overexpressing HUVECs, which showed a significant increase in the ability to form tubular structures and migrate (Fig. 6d–f). We inhibited Jagged 2 with neutralizing anti-Jagged 2 antibodies or increased the Dll4

promoter of the *Tgf β 1* gene. (g) Increased HOXD3 DNA binding in the *Tgf β 1* promoter in *Mgp*^{−/−} aortas as shown by ChIP assays. Data were analyzed by the Student's *t*-test. ***, *P* < 0.001. Error bars are standard deviation. ***, *P* < 0.001. Scale bar, 50 μ m. MGP, matrix Gla protein; HOXD3, homeobox D3; TGF β , transforming growth factor beta; BMP, bone morphogenetic protein; ALK, activin receptor–like kinase; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction.

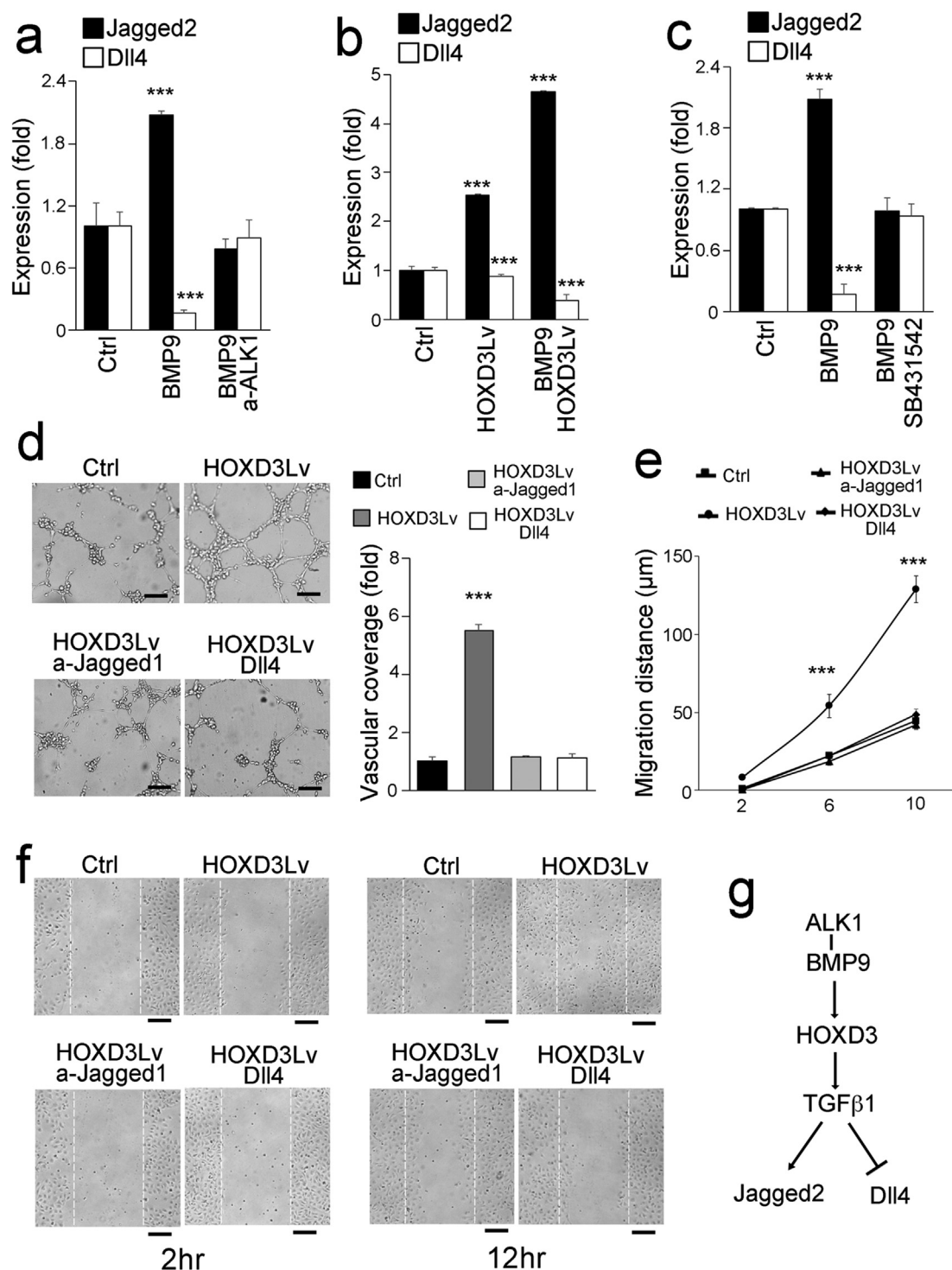


Fig. 6. HOXD3 balances BMP9-induced Notch signaling. (a) Expression of Jagged 2 and Dll4 in HUVECs after treatment with BMP9, with or without anti-ALK1 neutralizing antibodies. (b) Expression of Jagged 2 and Dll4 in HUVECs treated with BMP9, with or without infection of lentiviral vectors overexpressing HOXD3. (c) Expression of Jagged 2 and Dll4 in HUVECs after treatment with BMP9, with or without anti-ALK5 neutralizing antibodies. (d) Angiogenesis assay of HUVECs infected with lentiviral vectors overexpressing HOXD3 and treated with anti-Jagged 1 neutralizing antibodies or Dll4 ligands. The vascular coverage was measured and compared between different groups. (e–f) Migration assay of HUVECs infected with lentiviral vectors overexpressing HOXD3 and treated with anti-Jagged 1 neutralizing antibodies or Dll4 ligands. (g) Schematic diagram. Data were analyzed by the Student's *t*-test. ***, $P < 0.001$. Error bars are standard deviation. ***, $P < 0.001$. Scale bar, 50 μ m. HOXD3, homeobox D3; BMP, bone morphogenetic protein; ALK, activin receptor–like kinase; HUVECs, human umbilical vein endothelial cells; Dll4, delta-like 4.

activity with excess Dll4 in HOXD3-overexpressing HUVECs. The results showed that either inhibition of Jagged 2 or excess Dll4 limited the tube formation and the migratory capacity in the HOXD3-overexpressing HUVECs (Fig. 6d–f), suggesting that HOXD3 influences Notch signaling in angiogenesis (Fig. 6e).

Discussion

We have identified HOXD3 as a novel link between BMP and TGF β signaling in the vascular endothelium. We found that lack of MGP allowed for enhanced BMP9/ALK1 signaling to induce HOXD3 through direct action by pSMAD1 on the *Hoxd3* promoter, which in turn allowed for HOXD3 to enhance TGF β 1/ALK5 signaling through direct effects on the *Tgfb1* promoter. Thus, HOXD3 is in a key position to connect two pathways that are essential in vascular development and also to affect downstream Notch signaling.

In previous reports, HOXD3 was associated with the conversion from the quiescent endothelium to an invasive angiogenic phenotype [3–5], with some similarities to the endothelial-mesenchymal transitions that are known to occur in the *Mgp*^{−/−} mice [18]. However, it has also been reported that HOXD3 suppresses angiogenesis when targeted by micro-RNA miR-203a in the setting of hepatocellular carcinoma [6]. Our results add mechanistic details to how HOXD3 may effectuate BMP-stimulated changes in cells responsive to BMP9, through direct promoter effects or through induction of vascular growth factors or inhibitors.

Although our studies were performed in ECs, this system might be active in other vascular cells, such as the smooth muscle cells, which are highly dependent on TGF β signaling during development [25]. It is also possible that the reported differential expression of HOXD3 in venous versus arterial ECs reflects the balance between BMP and TGF β signaling in the different vascular beds. Therefore, it is not surprising that the HOXD3 also affected the expression of the Notch ligands Jagged 2 and Dll4, which have been associated with the stalk and tip cell phenotypes, respectively [26], supporting that HOXD3 is involved in the determination of EC fate.

Our previous studies showed that BMP4 is responsible for the induction of ALK1 in ECs through stimulation of ALK2 [27]. The action of BMP4 is counteracted by MGP, which directly binds and inhibits BMP4 as part of negative feedback regulation [15]. However, MGP does not bind BMP9 [28], implying that the suppressive effect of MGP on BMP9/ALK1 signaling is derived from the inhibition of BMP4 and in turn suppression of ALK1. This is

supported by our finding that limiting ALK1 suppressed the expression of HOXD3.

The HUVECs are a unique type of ECs, derived from the umbilical vein, which provide the embryonic liver with blood but receives little or no blood from the fetal liver. Expression of BMP9 has been shown mainly in the liver, which delivers BMP9 to various vascular beds through circulation [21]. However, it is unclear if BMP9 can cross the placenta, which may limit BMP9 activity in the endothelium of the umbilical vein. We found that the HUVECs express BMP9 (Supplemental Fig. 1). BMP9 was further induced by lack of MGP and suppressed by TGF β 1 (Supplemental Fig. 2), suggesting that BMP9 can also be provided endogenously by the HUVECs.

In summary, we provide novel evidence that BMP9 induces HOXD3 expression to mediate and regulate BMP9/ALK1 signaling and to provide links between TGF β and Notch signaling in angiogenesis.

Methods

Animals

Mgp[±] mice (B6·129S7-Mgptm1Kry/KbosJ) on C57BL/6J background were obtained from the Jackson Laboratory. The genotypes were confirmed by PCR [28], and the experiments were performed with generation F4–F6. Littermates were used as wild-type controls. All mice were fed a standard chow diet (Teklad Rodent Diet 8604, Envigo, Placentia, CA). The use of animals and all the experimental procedures were reviewed and approved by the University of California Los Angeles (UCLA) Chancellor's Animal Research Committee and conducted in accordance with the animal care guidelines set by UCLA. The investigation conformed to the National Research Council, *Guide for the Care and Use of Laboratory Animals, Eighth Edition* (Washington, DC: The National Academies Press, 2011).

Cell lines

HUVECs were obtained from Thermo Fisher Scientific (Waltham, MA) and cultured in Medium 200 phenol red free (Thermo Fisher Scientific), supplemented with Low Serum Growth Supplement (Thermo Fisher Scientific) at 37°C in a humidified chamber with 95% air and 5% CO₂.

RNA analysis

Real-time PCR analysis was performed as previously described [29]. GAPDH was used as a control gene. Primers and probes for human BMP9, HOXD3, MGP, TGF β 1, ALK1, ALK5, Jagged 1, Jagged 2, Dll4, and GAPDH were obtained from Applied Biosystems (Foster City, CA) as part of TaqMan® Gene Expression Assays.

Immunostaining

Tissue sections were fixed, processed, and stained as previously described [28]. Immunofluorescence was performed as previously described in detail [30]. Cultured cells were grown in chamber slides and fixed in 4% (weight/volume) paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100, blocked with 5% goat serum in phosphate-buffered saline (PBS), and stained using the same protocol as for tissue. We used specific antibodies for pSMAD1 (Millipore, St. Louis, MO), pSMAD2 (Thermo Fisher Scientific), HOXD3 (Santa Cruz Biotechnology, Dallas, TX), TGF β 1 and ALK1 (R&D Systems, Minneapolis, MN), and ALK5 (Sigma-Aldrich, St. Louis, MO). The nuclei were visualized with 4',6-diamidino-2-phenylindole (Sigma-Aldrich).

Immunoblotting

Total cell lysates were obtained by lysing the cells in radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Fisher Scientific). The protein concentration was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). The proteins were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (Thermo Fisher Scientific), transferred to nitrocellulose membrane, and then incubated with the primary antibodies. The antibodies included phosphorylated (p)-SMAD1/5/8 (diluted 1:500; Millipore), p-SMAD 2 (Thermo Fisher Scientific, diluted 1:500), HOXD3 and GAPDH (Santa Cruz Biotechnology, diluted 1:200), ALK5 (diluted 1:500; Sigma-Aldrich), and TGF β 1 and ALK1 (diluted 1:500; both from R&D Systems). After the membrane was washed three times with Tris-buffered saline with Tween 20, it was incubated with secondary antibodies, such as goat antirabbit antibodies or goat antimouse antibodies (diluted 1:1000; both from Cell Signal Technology, Danvers, MA) or donkey antigoat antibodies (diluted 1:5000; R&D Systems). The protein expression was normalized to β -actin in each sample.

Angiogenesis assay

Angiogenesis assays were performed as previously described [28]. Ninety-six–well plates were precoated with 50 μ l of Matrigel/well (BD Bioscience, San Jose, CA), which was allowed to polymerize for 30 min at 37 °C. HUVECs (4×10^4 cells/well) were resuspended in 100 μ l of culture medium and seeded in the Matrigel-coated 96-well plate. HUVECs were incubated for 6 h to allow formation of tube-like structures. The vascular coverage was measured and compared between conditions.

Chromatin immunoprecipitation assays

A commercially available ChIP assay kit (USB Corporation, Cleveland, OH) was used to perform ChIP assay, as per the manufacturer's protocol. Immunoprecipitation was performed using the same antibodies as were used for immunostaining. Real-time PCR was performed using the following primers for the TGF β 1 promoter: (F) 5'-GCTTCCTTGATCCCCTTCTT-

3', (R) 5'-GAACATGGATGGCAGACAAA-3' and (F) 5'-CCCCATCTTAATCCTTGGAC-3', (R) 5'-TAAAGCCACCACTTCCTCTCC-3', and for HOXD3 promoter: (F) 5'-TCCACTTAGGCACTTACTGGGT-3', (R) 5'-CAG-GAACTGCTCTGGTGAAGTC-3'. The conditions were as follows: 94°C denaturation, 55°C annealing, and 72 °C extension for 45 cycles.

Euthanasia

Euthanasia is required as an end point to allow collection and analysis of tissues. Mice are killed by isoflurane followed by cervical dislocation or intraperitoneal injection of sodium pentobarbital as recommended by the Panel of Euthanasia of the American Veterinary Medical Association.

Statistical analysis

The analyses were performed using GraphPad Instat®, version 3.0 (GraphPad San Diego, CA). Data were analyzed by either the unpaired 2-tailed Student's *t*-test or one-way analysis of variance with the Tukey's multiple comparisons test for statistical significance. Data represent mean \pm standard deviation. *P*-values less than 0.05 were considered significant, and experiments were repeated a minimum of three times.

Acknowledgments

Funding for this work was provided in part by National Institutes of Health (NIH) grants NS79353 (Y.Y.), HL139675 (Y.Y.), HL30568 (K.I.B.), and HL81397 (K.I.B.).

Author contributions

Y.Y. and K.I.B. supervised the experiments, analyzed data, and wrote the manuscript. L.W., J.Y., T.Y., D.Z., X.Q., X.W., and L.Z. performed experiments and data analysis.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2020.01.043>.

Received 17 October 2019;

Received in revised form 20 December 2019;

Accepted 27 January 2020

Available online 13 February 2020

Keywords:

endothelium;
bone morphogenetic protein;
Notch;
activin receptor-like kinase 1;
vascular development

Abbreviations used:

BMP, Bone morphogenetic protein; TGF β , Transforming growth factor β ; Hoxd3, Homeobox D3; ALK1, Activin receptor-like kinase 1; MGP, Matrix Gla protein; Dll4, Delta-like 4; EC, Endothelial cell; SMAD, Mothers Against Decapentaplegic Homolog; HUVECs, Human umbilical vein endothelial cells; ChIP, Chromatin Immunoprecipitation; vWF, von Willebrand factor.

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