Context-dependent signaling defines roles of BMP9 and BMP10 in embryonic and postnatal development

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Many important signaling pathways rely on multiple ligands. It is unclear if this is a mechanism of safeguard via redundancy or if it serves other functional purposes. In this study, we report unique insight into this question by studying the activin receptor-like kinase 1 (ALK1) pathway. Despite its functional importance in vascular development, the physiological ligand or ligands for ALK1 remain to be determined. Using conventional knockout and specific antibodies against bone morphogenetic protein 9 (BMP9) or BMP10, we showed that BMP9 and BMP10 are the physiologically, functionally equivalent ligands of ALK1 in vascular development. Timing of expression dictates the in vivo requisite role of each ligand, and concurrent expression results in redundancy. We generated mice (Bmp10<sup>β/β</sup>) in which the coding sequence of Bmp9 replaces that of Bmp10. Surprisingly, analysis of Bmp10<sup>β/β</sup> mice demonstrated that BMP10 has an exclusive function in cardiac development, which cannot be substituted by BMP9. Our study reveals context-dependent significance in having multiple ligands in a signaling pathway.

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Hereditary hemorrhagic telangiectasia (HHT) is a genetic disorder with multisystemic vascular dysplasia (1, 2). The underlying cause of the major clinical symptoms of HHT is a vascular anomaly referred to as arteriovenous malformation (AVM). HHT types 1 and 2 are the two major forms of HHT caused by heterozygous mutations in genes encoding endoglin (ENG) and ALK1 [also known as activin A receptor type II-like 1 (ACVR1L1)], respectively (3). ALK1, a type I receptor of the TGFβ receptor family, is predominantly expressed in endothelial cells (4). Genetic studies have revealed important roles of ALK1 during vascular development. In the absence of ALK1, mouse embryos die during midgestation with profound vessel dilation and severe AVM (5, 6). The importance of ALK1 signaling in vascular morphogenesis is also highlighted by its intimate link to other essential pathways in vascular biology, including Notch signaling (7).

Whereas genetic studies have established ALK1 signaling as an essential pathway regulating vascular development, the physiological ligand(s) responsible for ALK1 activation remains to be definitively determined. Early studies suggest that TGFβs signal through ALK1 and ALK5 in endothelial cells (8). David et al. however, show that bone morphogenetic protein 9 (BMP9) [also known as growth differentiation factor 2 (GDF2)] and BMP10, but not TGFβs, are capable of binding recombinant ALK1 (9, 10). BMP10 displays cardiac-specific expression (11). Bmp10<sup>−/−</sup> mice die between embryonic day 9.5 (E9.5) and E10.5 with profound defects in cardiac development (12). Interestingly, no defects in vascular development have been described in Bmp10<sup>−/−</sup> mice (12), leading to the speculation that the role of BMP10 may be limited to the cardiac tissue. On the other hand, it has been shown that BMP9, a liver-specific BMP, is present at significant levels in both mouse and human plasma (13, 14), suggesting that it could act systematically on the endothelium where ALK1 is expressed. Thus, existing evidence points to BMP9 as a potential physiological ALK1 ligand; however, its in vivo function has yet to be defined. In the current study, we seek to determine the in vivo functions of BMP9 and BMP10 using conventional knockout, function blocking antibody, as well as ligand replacement via knock in. Our data show that BMP10 but not BMP9 serves as a requisite endogenous ALK1 ligand during early embryonic vascular development, whereas both ligands serve a redundant role in postnatal vascular development. We also provide genetic evidence that defines two distinct functions of BMP10, with one to support vascular development via ALK1-dependent signaling in endothelial cells, which can be functionally substituted by BMP9 and the other to regulate heart development in a BMP10-exclusive manner. Our work suggests that BMP9 and BMP10 possess context-dependent signaling capacities.

Results and Discussion

BMP9 is dispensable for vascular development in mouse embryos and neonates. To investigate the potential role of BMP9 as an ALK1 ligand in vascular development, we characterized a mouse line in which the second exon encoding the receptor binding domain of BMP9 was replaced with a β-galactosidase reporter cassette (Fig. S1). To our surprise, Bmp9<sup>β/−</sup> mice were born at the expected Mendelian ratios, developed normally, and grew into normal fertile adults.

We first examined embryonic and extraembryonic vasculatures and found that loss of BMP9 did not result in any discernible vascular abnormalities (Fig. S2 C and D). We then assessed vascular development in early postnatal mice. Retinas collected from postnatal day 7 (P7) neonates were subjected to whole mount immunostaining with isocitrate B4 and alpha smooth muscle actin (αSMA). We found that the overall morphology and density of the retinal vasculature were comparable between wild type (WT), Bmp9<sup>β/−</sup>, and Bmp9<sup>−/−</sup> animals (Fig. S2E). In addition, we examined the neonate tracheas. Tracheal whole mounts were stained for platelet–endothelial cell adhesion molecule-1 (PECAM-1) and lymph vessel endothelial hyaluronan receptor-1 (LYVE-1) for blood and lymphatic vessels, respectively. Again, no noticeable difference was found between different genotype groups. These findings demonstrate that BMP9 is dispensable for vascular development in mouse embryos and neonates.

Neutralization of BMP10 causes neonatal vascular defect only when Bmp9 is mutated. Our finding that Bmp9<sup>−/−</sup> deficient animals did not exhibit any developmental and postnatal defects in vascular development suggested the presence of other ALK1 ligand(s).

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Among TGFβ family members, BMP10 and BMP9 share the highest sequence homology (9). To evaluate BMP10 as a potential ALK1 ligand, we took advantage of a BMP10-specific monoclonal antibody (Fig. S3). Neonatal mice were treated with anti-BMP10 from P2 through P7. We found that anti-BMP10 treatment had little effect in WT mice, but resulted in abnormal retinal vasculature in BMP9+/- and BMP9−/− animals (Fig. 1A). In BMP9−/-deficient mice, the expansion of the vascular plexus in the nerve fiber layer from the optic nerve head (ONH) was significantly delayed (Fig. 1A, Middle), and vascular density was markedly increased (Fig. 1A, Bottom). In addition, the affected blood vessels had reduced coverage of αSMA-positive mural cells (Fig. 1A, Middle). Quantifications of these changes demonstrated that the effect of anti-BMP10 was dependent on gene dosage of Bmp9, with greater severity in BMP9−/- animals relative to BMP9+/− animals (Fig. 1B and C).

Besides the retina, we also examined the trachea after neutralization of BMP10 (Fig. S4). We found that, in Bmp9−/- animals but not WT neonates, the tracheal blood vasculature was evidently disorganized. Moreover, the tracheal lymphatic vessels in Bmp9−/- animals were highly irregular and dilated. Therefore, simultaneous inhibition of BMP9 and BMP10 led to defective lymphatic vascular development in early postnatal mice as well. The phenotype is reminiscent of the effect of ALK1 fragment crystallizable region (ALK1Fc) that functions as a ligand trap to disrupt early postnatal lymphatic development (15). Our finding is also consistent with a recent report that BMP9 and BMP10 are critical for postnatal retinal vascular remodeling (16). Together, these results provide the initial in vivo evidence that both BMP9 and BMP10 are functionally redundant ALK1 ligands required for early postnatal vascular development.

Simultaneous Blockade of BMP9 and BMP10 with Neutralizing Antibodies Causes Vascular Development Defect in Postnatal Mice. To confirm the findings in Bmp9−/- mice, we generated a function-blocking monoclonal antibody selectively targeting BMP9 (Fig. S3). We analyzed P8 retinas and trachea of C57BL/6 mice following treatment with anti-BMP9 or anti-BMP10 as a single treatment, or in combination. Anti-BMP9 or -BMP10 alone had little effect on the retinal vascular development (Fig. 2A). However, combination treatment with both antibodies resulted in marked defects in the retinal vascular development (Fig. 2A), which were similar to those caused by ALK1Fc treatment (15), or those observed in Bmp9−/- mice treated with anti-BMP10 (Fig. 1). Similarly, neutralization of BMP9 or BMP10 alone did not affect the tracheal vasculatures. In contrast, combination treatment resulted in apparent defects both in blood and lymphatic vascular networks (Fig. 2B), comparable to those observed with ALK1Fc treatment (15). These results corroborate our findings in Bmp9−/- mice treated with the anti-BMP10 antibody and provide further evidence that BMP9 and BMP10 are functional ligands for ALK1.

BMP10 is a Circulating Factor. We developed ELISAs to specifically measure the protein levels of BMP9 and BMP10 in circulation. Consistent with previous reports (13, 14), we found the presence of BMP9 in mouse and human serum. Importantly, we also detected a significant level of BMP10, 0.5–2 ng/mL and 1–3 ng/mL in mouse and human serum, respectively. The presence of BMP9 and BMP10 in serum was further confirmed in a cell-based activity assay. Recombinant BMP9 or BMP10 induces SMAD family member 6 (SMAD6) expression in human umbilical vein endothelial cells (HUVECs), which can be specifically blocked by anti-BMP9 or anti-BMP10, respectively (Fig. S3A). We found that mouse serum was able to induce SMAD6 in HUVECs. Anti-BMP9 or anti-BMP10 alone only partially reduced SMAD6 induction. Combination of both antibodies, however, completely abolished the activity (Fig. 2C).

In the presence of conditioned medium from cultured human skin fibroblast (SF) cells, HUVECs growing in fibrin gels generate

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Fig. 1. Neutralization of BMP10 causes defective vascular development in Bmp9-deficient neonatal mice. (A) Fluorescence images of low (Top) and higher magnification (Middle and Bottom) of retinal vasculature. Neonatal mice were treated with PBS or anti-BMP10 (462732; R&D Systems) on P2 and P4, at 10 mg/kg. Whole mount P7 mouse retinas were stained with isoelectin B4 to visualize overall vasculature, and anti-αSMA for arteries. Dotted line at Middle marks the edge of retinal cup. (Scale bars: Top, 500 μm; Middle, 200 μm; Bottom, 25 μm.) (B) Quantification of vessel extension measured by the ratio of the distance from the ONH to the edge of the vascular network, over the distance from the ONH to the edge of retinal cup marked by the dotted line in A, Middle. Twelve representative measurements were taken from three treated retinas for each group. Results are shown as mean ± SD. (C) Quantification of vascular density. Total pixel counts from 12 representative images from three retinas of each condition. Results are expressed as mean ± SD *P < 0.05, **P < 0.002, ***P < 0.0005.
distinct sprouts (15, 17). Previously we showed that ALK1Fc was able to promote angiogenic sprouting, presumably by neutralizing ligands present under the assay condition (15). We found that anti-BMP9 or anti-BMP10 alone had little effect on HUVEC sprouting. In contrast, combination treatment with both antibodies was able to promote angiogenic sprouting, presumably by neutralizing ligands present under the assay condition (15). We found that anti-BMP9 (7A6), anti-BMP10 (462732; R&D Systems), or ALK1Fc were dosed every other day, at 10 mg/kg. Whole mount retinas of P8 mice were stained with isoelectin B4 and anti-α-SMA (Upper). (Scale bar: 200 μm.) Lower shows higher magnification views of boxed areas in Upper. (Scale bar: 100 μm.) (B) Tracheas of P8 mice were stained with PECAM-1 for blood vasculature (Upper), and LYVE-1 for lymphatic vasculature (Lower). (Scale bar: 250 μm.) (C) Serum induced up-regulation of SMAD6 expression in HUVECs in the presence of anti-BMP9 (7A6), anti-BMP10 (462732; R&D Systems), or both antibodies. Square dots represent each data point (n = 3). (D) HUVEC-coated beads were cultured in the presence of indicated agents. HUVEC sprouts were visualized by staining with DAPI and Alexa Fluor 488-phalloidin. (Scale bar: 175 μm.) Average sprout length from eight beads of each condition is shown. AU, arbitrary unit. Results are shown as mean ± SD. ***p < 0.0005.

**BMP10 Is Required for Early Embryonic Vascular Development.** Our findings described above provide compelling in vivo evidence that both BMP9 and BMP10 are physiological ALK1 ligands in postnatal mice. Given the strong vascular phenotype observed in Alk1-deficient mice (5, 6) and the apparent normal vascular development in Bmp9-deficient mice, we decided to reevaluate Bmp10-deficient mice for potential vascular defects (12). Bmp10-deficient embryo appeared to be normal at E8.5. By E9.5, however, the developmental defects became apparent. By E10.5, Bmp10-deficient embryos suffered from severe developmental arrest and were significantly smaller than wild-type embryos (Fig. 3A). Bmp10-deficient embryos had an enlarged pericardium (Fig. 3A, arrow), an indication of defective vascular development. Interestingly, similar developmental defects were observed in WT embryos after pregnant mice were treated with anti-BMP10 antibody (Fig. S3D).

In Bmp10-deficient yolk sac, the vitelline vessels were absent and vascular development was stalled at the primary capillary plexus stage (Fig. 3A). Normally, by E9.5, the dorsal aorta (DA) and cardinal veins (CV) have segregated into adjacent but distinct vessels (Fig. 3B). However, we found that Bmp10-deficient embryos developed AVM. The DA and CV were dilated and fused to form a single continuous channel (Fig. 3B). It is important to point out that the vascular defects identified in Bmp10-deficient embryos are reminiscent of the vascular phenotype resulting from the loss of Alk1 (5, 6), indicating that BMP10 is a key ALK1 ligand during early embryonic development.

**Temporal Expression of Bmp9 and Bmp10 in Early Embryonic Development.** In the current study, we found that in postnatal mice, BMP9 and BMP10 are functionally redundant for vascular development. However, why does loss of BMP10 but not BMP9 result in vascular defect during embryonic development? To address this question, we examined their temporal expression patterns. Whole mount X-gal staining of Bmp9−/− embryos revealed...
that the onset of liver-specific Bmp9 expression was around E9.75–10 (Figs. S2A and B and S5). In contrast, Bmp10 was detected as early as at E8.5 (Fig. S5), which coincided with the onset of Alk1 expression in mouse embryos (4). Therefore, during early embryonic development, there is a critical time window in which only BMP10 is present to activate ALK1. This temporal difference in the expression of Bmp9 and Bmp10 is consistent with the presence of developmental defects in Bmp10-deficient but not Bmp9-deficient mouse embryos.

BMP9 and BMP10 Are Interchangeable to Support Embryonic Vascular Development. BMP9 and BMP10 are highly homologous and both bind ALK1 with high affinity in vitro (9). In cultured endothelial cells, both ligands exhibit similar activity via signaling through ALK1 (9, 18, 19). In the current study, we show that BMP9 and BMP10 are functionally redundant in postnatal vascular development. These observations led us to postulate that BMP9 might be able to substitute BMP10 to support early vascular development.

To circumvent the temporal difference in the expression of Bmp9 and Bmp10, we created a Bmp9 knock-in mouse line (Bmp10+/9) in which the Bmp10 coding region was replaced by that of Bmp9. Both Bmp9 and Bmp10 genes have two exons with similar genomic structure. To minimize the impact on gene regulation, only the coding sequence of Bmp10 was substituted (Fig. 3C). Heterozygous Bmp10+/9 mice were viable and had no discernible developmental defects. In adult heterozygous Bmp10+/9 mice, the expression pattern of ectopic Bmp9 mirrored that of endogenous Bmp10. Both ligands were only detected in the right atrium, but not other compartments of the heart (Fig. 3D). In addition, in homozygous Bmp10+/9 embryos, the expression pattern of Bmp9 was indistinguishable from that of Bmp10 in WT embryos, both being expressed in trabecular myocardium, but not in compact myocardium (Fig. 3E). We further characterized the heart-specific Bmp9 transcript from Bmp10+/9 mice by RT-PCR followed by sequencing, confirming that the expected Bmp9 transcript was produced from the knock-in allele.

We analyzed the development of homozygous Bmp10+/9 embryos. Up to E16.5, Bmp10+/9 embryos did not exhibit any gross developmental defects (Fig. 3F and Fig. S6). This was in sharp contrast to Bmp10-deficient embryos, which displayed overt developmental defects by E9.5. Unlike the loss of Bmp10, the vitelline vessels in the Bmp10−/− yolk sac were apparently normal (Fig. 3F). Furthermore, Bmp10+/9 embryos had distinct dorsal aorta and cardinal vein, lacking the observed AVM in Bmp10-deficient embryos. These findings indicate that BMP9 and BMP10 are functionally interchangeable to support ALK1-dependent early vascular development.

Dual Functions of BMP10 in Early Embryonic Development. Interestingly, intercrossing of heterozygous Bmp10+/9 mice yielded no live-born homozygous Bmp10−/− offspring. Close examination revealed that, starting around E16.5, Bmp10−/− embryos were affected by apparent hemorrhage and edema, with increased severity in older embryos (Fig. S6A). Bmp10 exhibits a heart-specific expression pattern and early studies have suggested its role in cardiac development and homeostasis (12). Because early vascular development in Bmp10−/− embryos was largely normal, we suspected that the late vascular phenotype might be secondary and reflect defective heart development. To evaluate this possibility, we examined cardiac development in Bmp10−/− embryos. At E14.5, although the gross appearance of Bmp10−/− embryos was normal, the heart had readily shown clear signs of developmental defects. Bmp10−/− hearts were hypoplastic with significantly thinner ventricular wall and marked pericardial edema (Fig. 4A). At E17.5, the gross morphology of the heart became overtly abnormal, with

Fig. 3. BMP10 is required for early vascular development and this role is interchangeable with Bmp9. (A) Gross morphology of E10.5 yolk sac and embryos of control and Bmp10+/−. (B) Paraffin section of E9.5 embryos stained with PECAM-1 for blood vessels. (C) Targeting vector was designed to make the Bmp9 knock-in allele. (D) RT-PCR expression analysis of Bmp9 and Bmp10 in adult heterozygous Bmp9 knock-in (Bmp9+/9) mice. (E) In situ hybridization on embryonic hearts of WT and homozygous Bmp9 knock-in (Bmp9−/−) embryos. (F) E10.5 yolk sac and embryos of control and Bmp10+/9 animals.
enlarged size and altered shape (Fig. S6B). A majority of Bmp10<sup>+/9</sup> hearts also presented with pronounced ventricular septal defects (Fig. 4A and Fig. S6B). The developmental defects in Bmp10<sup>+/9</sup> hearts are reminiscent of previous findings in Bmp10-null embryos (12). Assessment of immune reactivity to anti-Ki67 antibody (a marker for cell proliferation) in myocardium revealed significantly reduced cell cycle activity in Bmp10<sup>+/9</sup> myocardium (Fig. 4A), further supporting the role of BMP10 in promoting cardiomyocyte proliferation and myocardial growth during ventricular development (12). These results demonstrated that ectopic expression of Bmp9 in the heart failed to fully compensate for the loss of Bmp10 in the developing heart, which was in contrast to the ability of Bmp9 to restore early vascular development in the absence of Bmp10. Therefore, genetic replacement of Bmp10 with Bmp9 allowed defining two distinct functions of BMP10 during development with one to support early vascular development and the other to regulate heart development. It is worth pointing out that the apparent edema observed in late-stage Bmp10<sup>+/9</sup> embryos suggests lymphatic defects. Future studies are required to further characterize the underlying defects.

We showed that the expression pattern of Bmp9 in Bmp9 knock-in mice faithfully reflected that of Bmp10. Sequencing analysis indicated that Bmp9 transcript was correctly produced and processed from the knock-in allele. Furthermore, heart-specific expression of Bmp9 was sufficient to support early embryonic vascular development. These observations raise the possibility that BMP9 and BMP10 have distinct signaling capacities, especially in nonendothelial cells. Ligands of TGFβ family signal by forming a ternary protein complex that comprises dimeric ligands, two type I receptors, and two type II receptors. Ligand binding results in the transphosphorylation and activation of type I receptor by the constitutively active type II receptor. A recent report shows that BMP9, but not BMP10, displays a significant discrimination in type II receptor selection (20). Among the three type II receptors [BMP receptor II (BMPRII) and activin type II receptors A and B (ActRIIA and ActRIIB)] implicated in BMP9 and BMP10 signaling, BMP9 exhibits striking bias toward ActRIIB. Because in vitro, BMP9 and BMP10 only bind ALK1, but not other type I receptors, with high affinity (9), this raises the interesting possibility that BMP9 might have limited signaling capacity in cells where the expression level of ALK1 and ActRIIB are limited (Fig. 4B). Our observation that BMP9 is unable to substitute for BMP10 in regulating heart development is consistent with the broader specificity of BMP10 in interacting with multiple type II receptors.

Conclusions

Our current study exemplifies the power of combining genetic approaches with selective pharmacological tools such as function-blocking antibodies. The use of a BMP10-specific antibody allowed us to overcome the limitation of embryonic lethality associated with loss of Bmp10 to reveal its role in postnatal vascular development. It has been shown that ALK1Fc functions as a ligand trap to cause vascular phenotypes similar to those observed in Alk1- and Eng-deficient mice (15). Although in vitro ALK1Fc binds and effectively blocks the activity of both BMP9 and BMP10, in principle we cannot rule out that ALK1Fc might negate the activity of another unknown ligand(s) in vivo. In the current study, the use of ligand-specific antibodies and targeted gene disruption allowed us to definitely conclude that BMP9 and BMP10 are the physiological ligands for ALK1 signaling.

Compared with the conventional gene targeting approach, the gene replacement strategy we used provides deeper insight into the role of BMP10 during embryonic development. Furthermore, it offers important in vivo evidence that BMP9 and BMP10 possess context-dependent signaling capacities, as suggested by the in vitro analysis of BMP9 and BMP10 interactions with ALK1 and type II receptors (20). Besides the ALK1 pathway studied in the current work, many important signaling pathways rely on multiple ligands. The strategy described in the current study could be used to reveal the potentially unique functions inherent to individual ligands.

In addition to its link to a genetic disorder, ALK1 is also implicated in tumor angiogenesis (10, 21, 22). Agents that block the VEGF pathway have been extensively investigated in preclinical tumor models and some of them have been clinically validated (23, 24). Different tumors, however, exhibit varied sensitivity to VEGF blockade. Therefore, additional strategies are needed for targeting tumor angiogenesis (25, 26). Defining BMP9 and BMP10 as the physiological ligands for ALK1 offers important guidance for the development of unique diagnostic and therapeutic agents.

Materials and Methods

Neutralizing Antibodies. Anti-BMP9 monoclonal antibody (clone 7A6) was raised in Bmp9 KO mice using standard mouse hybridoma techniques.
Recombinant mouse BMP9 protein (R&D Systems) was used as the immunogen. A neutralizing antibody was selected based on its ability to inhibit SMAD6 induction by BMP9 in HUVECs. The neutralizing anti-BMP10 antibody (clone 462732) was purchased from R&D Systems. A hamster anti-BMP10 (clone 40G4S) was also generated using standard hybridoma technique.

**Generation of BMP9 KO and BMP9 Knock-In Mice.** To make BMP9 KO mice, a gene targeting vector was designed to replace exon2 of BMP9 with a β-galactosidase (βgeo)purumycin cassette. The targeting construct was electroporated into 129SvEvBrd (Lex-2) ES cells. Cells carrying the targeted allele were injected into C57Bl/6 albino blastocysts. The resulting chimeras were mated to C57Bl/6 albino females, and the heterozygous offspring were backcrossed onto C57Bl/6 backgrounds. Gene targeting and initial mouse breeding was performed at Lexicon Pharmaceuticals, Inc.

To generate the BMP9 knock-in mice, a gene-targeting vector was made using a combination of recombineering (27) and standard molecular cloning techniques. Additional details are provided in *SI Materials and Methods*.

**Neonatal Vascular Analysis.** Neonatal mice were injected i.p. with mAbs or fl6171. 408.438(7070):922.

**Development**

**Values** 27325.184.112.

BMP9 Blood 109(5):1953110.

KO mice, 400 HUVECs per bead. About 200 HUVEC-coated 331.2231.

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**Neonatal Vascular Analysis.** Neonatal mice were injected i.p. with mAbs or ALK1Fc (15) at 10 mg/kg. The treatment schedules are specified in the figure legends. Retinas were dissected from the eye following overnight fixation in 4% (wt/vol) paraformaldehyde (PFA) at 4 °C. Retina was blocked with 10% (vol/vol) Triton X-100, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.1 mM MnCl2 in PBS (pH 6.8). Retina was then washed and stained with Alexa Fluor 488 streptavidin (Molecular Probes), and Cy3 antisense muscle actin (Sigma). Images of flat-mounted retina were taken by standard and confocal fluorescence microscopy. For analysis, isolated retina was fixed with 4% (wt/vol) paraformaldehyde (PFA) at 4 °C with 25 mM biotinylated isoelectin B4 (Sigma) in PBLEC (1% Triton X-100, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.1 mM MnCl2 in PBS [pH 6.8]). Retina was then washed and stained with Alexa Fluor 488 goat antihamster (Jackson ImmunoResearch) and Alexa Fluor 594 goat anti-rabbit (Invitrogen). Whole-mounted retina was analyzed by confocal fluorescence microscopy.

**Mouse Embryo Analysis.** Gestational age was determined by the date of the copulation plug. For embryos younger than E10.5, gestational age was further confirmed by the number of somites. Sibling pairs were used for comparison. Whole mount immunohistochemistry of embryo and yolk sac was carried out as described (28). Rat anti-mouse PECAM (BD Pharmingen) was used as the primary antibody. Donkey anti-rat HRP (Jackson) were used as secondary antibody. Color was developed using the metal enhanced DAB substrate kit (Pierce). Stained embryos were embedded in paraffin and then sectioned at 10-μm thickness. Whole mount staining for iGeo activity was performed as described (29). Embryos were fixed for 1 h in fresh 4% PFA in PBS at 4 °C. Fixed embryos were washed three times in wash buffer and stained for 16–24 h in the dark with 1 mg/mL X-gal. After staining, embryos were briefly washed and postfixed in 4% PFA for 5 h.

**Cardiac Histological Analysis and in Situ Hybridization.** Embryonic hearts were isolated and fixed with 4% PFA in PBS, paraffin embedded, and sectioned (7 μm), followed by hematoxylin and eosin (H&E) staining. In situ hybridization was performed as previously described (12). BMP9 and BMP10 antisense probes were labeled with digoxigenin-UTP using the Roche DiG RNA Labelling system according to the manufacturer’s guidelines.

**HUEV C Fibrin Gel Bead Assay.** Details of the HUEVC fibrin gel bead assay have been described (17). Briefly, Cytox 3 beads (Amer sham Pharmacia Biotech) were coated with 350–400 HUEVCs per bead. About 200 HUEVC-coated beads were imbedded in fibrin clot in one well of 12-well tissue culture plate. Assays were terminated between day 5 and 7 for immunostaining and imaging. To visualize HUEVC sprouts, fibrin gels were fixed overnight in 2% PFA, and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and Alexa Fluor 488-phalloidin (Invitrogen). Images of the beads were captured with standard fluorescence microscopy.

**Statistics.** If not otherwise mentioned, averages were calculated from triplicate experiments. Groups were compared using a two-tailed, unpaired Student t test. P values <0.05 were considered significant.

**Animal Studies.** All studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, revised 1985). The institutional animal care and use committee approved all animal protocols.

Additional and more detailed experimental procedures can be found in Supporting Information.

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