Distinct Signaling Pathways Regulate Sprouting Angiogenesis from the Dorsal Aorta and Axial Vein

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Abstract

Angiogenesis, the formation of new blood vessels from preexisting vessels, is critical to most physiological processes and many pathological conditions. During zebrafish development, angiogenesis expands the axial vessels into a complex vascular network that is necessary for efficient oxygen delivery. Although the dorsal aorta (DA) and the axial vein (AV) are spatially juxtaposed, the initial angiogenic sprouts from these vessels extend in opposite directions, suggesting that distinct cues may regulate angiogenesis of the axial vessels. In this report, we found that angiogenic sprouts from the DA are dependent on Vegf-A signaling, and do not respond to Bmp signals. In contrast, sprouts from the AV are regulated by Bmp signaling independent of Vegf-A signals, suggesting that Bmp is a vein-specific angiogenic cue during early vascular development. Our results support a paradigm, whereby different signals regulate distinct programs of sprouting angiogenesis from the AV and DA, and suggest that signaling heterogeneity contributes to the complexity of vascular networks.

The DA and AV form a primitive circulatory loop, and subsequent angiogenesis from these vessels is essential to generate the complex vascular networks found in vertebrates. In zebrafish, the initial sprouts from the DA project dorsally to form the intersegmental arteries

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(ISAs)[1] (arrows, Suppl. Fig. S1a), while those from the posterior AV extend ventrally (arrowheads, Suppl. Fig. S1a) to form a honeycomb-like network termed the caudal vein plexus (CVP), which is composed of a dorsal and ventral vein with interconnecting vessels (Suppl. Fig. S1a). Since the neighboring axial vessels extend angiogenic sprouts in opposite directions and form distinct vascular networks, we hypothesized that the DA and AV respond to different angiogenic stimuli.

The Vascular Endothelial Growth Factor-A (Vegf-A) signaling cascade is a critical angiogenic stimulus for many vascular beds[2], so we first assessed the role of Vegf-A in regulating sprouting angiogenesis from the axial vessels. Co-injection of morpholinos (MOs) against two Vegf-A receptors in zebrafish, *kdrl* and *kdr*[3], caused severe vascular defects. The DA and the CV incompletely segregate[4] (Fig 1a), endothelial cell apoptosis was significantly increased[5] (Suppl. Fig. S1b), and ISA sprouts were blocked[5] (Fig. 1a). While the percentage of segments (the area defined by two adjacent somite boundaries) containing an ISA was drastically reduced, the percentage containing a CVP was largely unaffected in *kdrl*/*kdr* morphants (Fig. 1b) (see Materials and Methods for quantification specifics). The venous sprouts still formed a primitive plexus in *kdrl*/*kdr* morphants, and only displayed marginal defects in branching (Fig. 1 and Suppl. Fig. S1c). This vascular network was unstable and ultimately regressed, as previously reported[4]. While our data corroborate the role of Vegf-A signaling in regulating ISA formation and endothelial cell stability[3], they suggest that another angiogenic stimulus regulates sprouting from the AV.

To identify the angiogenic signal required for sprouting from the AV, we analyzed the expression of components from several signaling pathways (data not shown), and found that Bone Morphogenetic Protein (Bmp) pathway components were selectively expressed in the developing CVP. Whole mount *in situ* hybridization indicated that the *bmp2b* ligand was highly expressed within the CVP and surrounding tissue during plexus formation (26–32 hours post-fertilization (hpf)), and expression subsided as the CVP stabilized at 38hpf (Fig. 1c and Suppl. Fig. S1d). In addition, two Bmp type II receptors, *bmpr2a* and *bmpr2b*, were strongly expressed in the endothelial cells of the CVP at 26, 32, and 38hpf consistent with previous studies[6] (Fig. 1c and Suppl. Fig. S1d).

Bmp can function as a context-dependent pro-angiogenic cue[7]. Upon ligand binding, Bmp type II receptors phosphorylate Bmp Type I receptors, which in turn activate Smad and/or MAP kinase signaling[8]. To test whether Bmp signaling regulates sprouting of the AV, we manipulated expression of Bmp pathway components in the developing zebrafish using a heat shock promoter (*hsp70l*)[9]. To determine the expression profile of *hsp70l*, we heat-shocked *Tg(hsp70l:GFP)* embryos at 25hpf and found that GFP was expressed in most tissues and cell types (Suppl. Fig. S2a). We next analyzed the effects of decreased Bmp activity on sprouting from the AV by over-expressing *noggin3*, an endogenous inhibitor of Bmp signaling[10]. Control embryos heat-shocked at the onset of plexus formation (25hpf) showed no apparent vascular abnormalities (Fig. 2a and Suppl. Movie S1). In contrast, heat-shocked *Tg(hsp70l:noggin3)* embryos displayed CVP with aberrant sprouts that failed to make proper connections with neighboring sprouts, but showed no ISA defects (arrows, Fig. 2a, Suppl. Fig. S3a, and Suppl. Movie S2). Similar results were also observed in *Tg(hsp70l:dnbmprI-GFP)* embryos that expressed a dominant negative Bmp receptor type I

GFP fusion (DNBmprI-GFP) when heat-shocked (Suppl. Fig. S2b). Since CVP patterning was perturbed while ISA patterning was largely unaffected, these results suggest that decreased Bmp signaling selectively affects vessel patterning from the AV.

We next asked whether increased Bmp signaling could induce angiogenesis. *bmp2b* expression was increased in heat-shock treated *Tg(hsp70l:bmp2b)* embryos at the onset of CVP formation. *bmp2b* over-expression induced ectopic sprouts along the AV, with the most robust ectopic sprouting occurring in the CVP (arrowheads, Fig. 2a, Suppl. Fig. S3b, and Suppl. Movie S3). Bmp-induced ectopic sprouts extended from the AV and migrated between the epithelial surface and the somite boundary, forming an additional plexus in a region that is avascular in wild-type (WT) embryos (arrowheads, Suppl. Fig. S4a). The Bmp-induced plexus highly expressed a venous marker, *dab2*, indicating that it has venous identity (Fig. S4b). Although Bmp over-expression induced robust sprouting from the AV, ectopic sprouts were never observed from the DA (Fig 2a). To further delineate the specificity of Bmp signaling, WT and *Tg(hsp70l:bmp2b)* embryos were heat-shocked at 2.5dpf, when ventral sprouts from the AV form the subintestinal vein plexus (SIVP) (arrows, Suppl. Fig. S4c). The SIVP in *bmp2b* over-expressing embryos was shifted dorsally (arrows, Suppl. Fig. S4c) and contained ectopic vessels (arrowhead, Suppl. Fig. S4c), suggesting that the SIVP is also responsive to Bmp signaling. These data indicate that sprouting angiogenesis from the AV during early development is uniquely dependent on Bmp signaling.

To assess the cellular effects of Bmp signaling on venous endothelial cell behavior, we performed time-lapse imaging. WT embryos formed a honeycomb-like plexus by 32hpf, and this plexus began to retract filopodia and stabilize by 35hpf (Fig. 2b and Suppl. Movie S1). However, *Tg(hsp70l:noggin3)* embryos contained atypical angiogenic sprouts that failed to make connections and never formed a proper plexus (Fig. 2b and Suppl. Movie. S2). In contrast, *Tg(hsp70l:bmp2b)* embryos contained ectopic endothelial sprouts. These ectopic sprouts branched and sprouted from the dorsal vein of the CVP as early as 6.5 hours after heat-shock treatment (32hpf) and rapidly migrated dorsally (Fig. 2b and Suppl. Movie S3).

To better characterize the *noggin3* and *bmp2b* over-expression phenotypes, we counted venous endothelial cell nuclei and performed branch point analyses in WT, *noggin3*, and *bmp2b* over-expressing embryos. While the number of venous endothelial cells in the CVP remained relatively unchanged in *noggin3* over-expressing embryos, we observed a slight but significant increase in endothelial cell numbers in *bmp2b* over-expressing embryos (Supp Fig S3c). Venous branch points, however, were significantly altered in both *noggin3* and *bmp2b* over-expressing embryos (Suppl. Fig. S3d). We found that the number of branch points was decreased more than 7 fold by *noggin3* over-expression, and increased approximately 2.5 fold by *bmp2b* over-expression (Suppl. Fig. S3d). In addition, *bmp2b* over-expression caused a significant increase in the number of filopodia (Suppl. Fig. S4d–e), and randomized their direction of extension (Suppl. Fig. S4f). Taken together, our data indicate that Bmp is a pro-angiogenic cue that regulates angiogenesis in the AV.

To investigate whether the Bmp type II receptors expressed in the developing CVP regulate Bmp-mediated angiogenesis, we analyzed *bmpr2a* or *bmpr2b* morphants (Fig. 3a–d and

Suppl. Fig. S5). While the number of arterial sprouts did not differ significantly from control embryos, sprouts from the AV were significantly reduced in *bmpr2a* and *bmpr2b* morphants (Fig. 3a–b and Suppl. Fig. S5c). Moreover, knock-down of *bmpr2a* or *bmpr2b* in *bmp2b* over-expressing embryos inhibited formation of ectopic sprouts (Fig. 3c–d and Suppl. Fig. S5d). Therefore, Bmpr2a and Bmpr2b regulate Bmp-mediated angiogenesis from the AV.

Considering the expression and function of *bmpr2a* and *bmpr2b* in CVP formation, it is likely that Bmp activation is required in endothelial cells. To investigate this hypothesis, we generated mosaic embryos by injecting either *kdrl:GFP* or *kdrl:DNBmprI-GFP* in the *Tg(kdrl:mCherry)* background. The resulting embryos contained patches of endothelial cells that strongly expressed *GFP* or *DNBmprI-GFP*. The *GFP*-expressing control cells extended venous sprouts, which made connections and formed a honeycomb-like plexus (Fig. 3e and Suppl. Movie S4). In contrast, the *DNBmprI-GFP*-expressing cells were unable to extend sprouts from the AV, and they failed to connect with neighboring endothelial cells to form a honeycomb-like plexus (Fig. 3f and Suppl. Movie S5).

The segments that contained *DNBmprI-GFP*-expressing cells had fewer branch points than *GFP* expressing control cells (Fig. 3g), indicating that Bmp signaling within endothelial cells is important during branching morphogenesis. In addition, the frequency with which the branches connected to form a plexus was significantly reduced in *DNBmprI-GFP*-expressing cells, suggesting that Bmp signaling within endothelial cells is critical for the formation of endothelial networks (Fig. 3h). Taken together, our results indicate that Bmp-mediated angiogenesis requires Bmp activation in endothelial cells.

Bmp signaling activates the Smad signaling cascade and/or alternative MAP kinase signaling cascades such as Erk and p38[11,12]. To delineate the downstream factors critical for Bmp-mediated angiogenesis, we first analyzed the activity/phosphorylation status of Smad1/5/8 (R-Smads) and Erk. Activated R-Smads and Erk were present within the ectopic sprouts from the AV (Suppl. Fig. S6a–b). To assess the function of R-Smad and Erk signaling in Bmp-mediated angiogenesis, we blocked the activity of R-Smad or Erk by treating embryos with small chemical inhibitors. To inhibit the R-Smad signaling cascade, we used DMH1, which inhibits Alk2/3 and selectively abrogates activation of R-Smads without affecting MAP kinase activity[13]. In addition, we inhibited the p38 pathway with SB203580, and the Erk pathway with either U0126 (data not shown) or SL327. While both arterial and venous angiogenesis was unaffected by treatment with DMSO or the p38 inhibitor, inhibition of R-Smad activation selectively blocked formation of the CVP without affecting ISAs, and inhibition of Erk activity blocked formation of both the CVP and ISAs (Fig. 4a–b). Moreover, inhibiting R-Smad or Erk activation in Bmp over-expressing embryos efficiently inhibited the percentage of segments with ectopic vessels, while p38 inhibition had no effect on the percent of segments with ectopic vessels (Fig. 4c–d). Interestingly, the Erk inhibitor also drastically attenuated the length and progression of the ectopic sprouts (Fig. 4e). Collectively, these results suggest that R-Smad activation selectively regulates venous sprouting angiogenesis, and Erk (but not p38) activation is involved in the progression of Bmp-mediated venous sprouts as well as arterial sprouts.

Since activation of the Bmp signaling cascade transcriptionally regulates multiple genes and pathways, we analyzed the transcriptional levels of important regulators of angiogenesis using quantitative RT-PCR. Transcription levels of *Tg(hsp70l:bmp2b)* embryos were compared to WT at 2 and 5 hours post heat-shock induction of *bmp2b*. *id2a*, a downstream transcriptional target of Bmp signaling, was used as a positive control. *vegfa*, *vegfc* (also a major stimulus of lymphangiogenesis[14]), *vegfr3/flt4* (a venous marker and receptor for Vegf-C), and *dll4* (an arterial marker and tip cell marker[15,16]) were also tested. Bmp over-expression upregulated *id2a* by over 3-fold at 2 hours post heat-shock, while *vegfa*, *vegfc*, *flt4*, and *dll4* were either marginally affected or not affected at all (Suppl. Fig. S7). In addition *vegfa*, *vegfc*, *flt4*, and *dll4* transcript levels were unaffected 5 hours post heat-shock induction of *bmp2b* (Suppl. Fig. S7).

To test the physiological relevance of the moderate increase in *vegfa* transcription at 2 hours post heat-shock, Bmp over-expression was induced in embryos lacking Vegf receptors. Co-injection of the *kdrl/kdr* MOs resulted in a single AV at 2dpf (Fig. 5a). Despite the severely disrupted vascular network, Bmp-induced ectopic blood vessels were unaffected in *kdrl/kdr* morphants, demonstrating that Bmp is capable of inducing angiogenesis when Vegf receptors are inhibited (Fig. 5a–b). We next analyzed the effects of Bmp and Vegf-A small molecule inhibitors during sprouting angiogenesis of the axial vessels[12]. Addition of dorsomorphin, a chemical inhibitor of both the Bmp and Vegf-A signaling pathways, effectively inhibited vessels from the DA and AV and blocked Bmp-induced ectopic vessels (Suppl. Fig. S8). DMH4, an inhibitor of Vegf-A signaling, preferentially blocked vessels from the DA and had no effect on Bmp-induced ectopic vessels, while DMH1, an inhibitor of Bmp signaling, selectively inhibited vessels from the AV and disrupted Bmp-induced ectopic vessels (Suppl. Fig. S8). Taken together, these findings demonstrate that Bmp is the major stimulus for sprouting angiogenesis from the AV, and that Vegf-A is the major stimulus for sprouting from the DA. Secondly, these results suggest that Bmp mediates angiogenesis independent of a significant contribution from Vegf-A signaling.

To compare the angiogenic effects of Bmp and Vegf-A, we induced over-expression of *bmp2b* or *vegfa₁₂₁* by heat-shock treatment of *Tg(hsp70l:bmp2b)* or *Tg(hsp70l:vegfa₁₂₁)* transgenic lines, respectively. As expected, *bmp2b* over-expression induced robust ectopic sprouts along the AV, but not from the DA (Fig. 5c). In contrast, *vegfa₁₂₁* over-expression did not induce ectopic sprouts from the AV, but increased sprouting along the DA was observed (Fig. 5c). The distinct angiogenic responses between *bmp2b* over-expressing embryos and *vegfa₁₂₁* over-expressing embryos demonstrate that Bmp is a distinct and potent pro-angiogenic factor.

Taken together, our findings support a paradigm whereby Bmp signaling mediates venous angiogenesis, while Vegf-A signaling directs arterial angiogenesis. In our model, this differential response to angiogenic stimuli permits neighboring venous and arterial vessels to extend distinct angiogenic sprouts and form non-overlapping vascular networks (Fig. 5d). The venous sensitivity observed during Bmp-mediated angiogenesis may be provided by the notochord, which lies above the DA and expresses Bmp antagonists that inhibit blood vessel growth[17,18]. Collectively our results suggest a model of Bmp mediated angiogenesis in which Bmp2b binds Bmpr2a/b and Alk2/Alk3 hetero-tetrameric receptor complexes in

venous endothelial cells and activates R-Smad and Erk, which elicits angiogenic responses that include sprout migration and fusion (Fig. 5e).

The zebrafish embryo contains a relatively simple and streamlined vascular system, and this simplicity allows for elucidation of binary choices that likely underlie vascular development in more complex organisms. It will be important to determine if there is a similar role for Bmp signaling during mammalian development and tumor angiogenesis. Although published work in mammalian systems does not identify a selective requirement for Bmp signaling in venous angiogenesis, mammalian vascular systems are more complex, and the requirement for BMP signaling in early development makes specific interrogation of later requirements difficult[19–22]. In addition, several types of carcinomas express high levels of BMP growth factors[23–25], and anti-angiogenesis cancer drugs that singularly antagonize VEGF-A activity are only partially effective[26]. Therefore, future studies that target both Bmp and Vegf-A signaling may be more successful at manipulating blood vessel growth.

Methods

Zebrafish husbandry

Zebrafish (*Danio rerio*) embryos were raised as previously described[27]. The following transgenic lines were used: *Tg(fli1:nEGFP)^{y7}* [28], *Tg(kdrl:GFP)^{s843}* [29], *Tg(kdrl:ras-mCherry)^{s896}* [30], *Tg(hsp70l:bmp2b)^{fr13}* [31], *Tg(hsp70l:noggin)^{fr13}* [31], *Tg(hsp70l:dnbmprI-GFP)^{w30}* [32], and *Tg(hsp70l:vegfaa$_{121}$;cmlc2:EGFP)^{nc2}* (this study).

In situ hybridizations and immunohistochemistry

Whole mount *in situ* hybridization was performed as previously described[33,34] to probes for *bmp2b*, *bmpr2a*, *bmpr2b*, and *dab2* were synthesized as previously described[6], and documented with a Leica MF16 microscope. For transverse sections, embryos were mounted in 4% agarose, embedded in paraffin, and sectioned into 8, 7, and 5μm slices respectively. Fast red staining was used to visualize tissue morphology.

Immunohistochemistry was performed as previously described[29]. Following antibodies were used: anti-Caspase3, cleaved (Cat#:PC679, Calbiochem), β-tubulin (Cat#:61053, BD Transduction Laboratories) at 1:200, and Alexa Fluor secondary antibodies (Invitrogen) at 1:400. To sagittally mount embryos, the head and yolk were removed and the trunk was covered in 1% low melt agarose and sealed with a cover slip. For transverse sections, embryos were mounted in 4% agarose and sectioned on a Leica VT 1000s vibratome.

Heat-shock treatment

Tg(hsp70l:noggin), Tg(hsp70l:bmp2b), Tg(hsp70l:vegfaa$_{121}$;cmlc2:GFP), and *Tg(hsp70l:dnbmprI-GFP)* embryos were heat-shocked 25–26hpf for 30minutes at 42°C. *Tg(hsp70l:noggin)* and *Tg(hsp70l:bmp2b)* embryos were genotyped by PCR, and *Tg(hsp70l:vegfaa$_{121}$;cmlc2:GFP)* and *Tg(hsp70l:dnbmprI-GFP)* embryos were identified by the expression of GFP.

Quantification

To quantify and compare arterial and venous angiogenesis in Fig. 1b, 3b, 3h, 4b, 5b, Suppl. Fig. S6a, S14a–b, and S18b, we calculated the percentage of segments that form an angiogenic vessel from the DA (ISA) or from the AV (CVP) between 36–40hpf. Each segment is defined as the area on the A-P axis between two adjacent somite boundaries. The first 12 segments starting at the end of the yolk extension (roughly corresponding to the 14th to 26th somite) were analyzed. To quantify arterial angiogenesis (red bars), each segment that contained an ISA (at the anterior somite boundary) that reached the DLAV was given a value of 1, while each segments that lacked an ISA was given a value of 0. Similarly, to quantify venous angiogenesis (blue bars), each segment that contained a CVP with a fused ventral vein (therefore, completed the CV remodeling) was given a value of 1, and segments that lacked a fused ventral vein in the CVP were given a value of 0. These values were then used to calculate the percentage of segments with either ISA (red bars) or CVP (blue bars).

To quantify ectopic vessels in *bmp2b* over-expressing embryos in Fig. 3d, 4d, 5b, Suppl. Fig. S6b, S14b, and S18c embryos were examined between 44–50hpf. Since the ectopic sprouts and pairs of ISAs formed on in both the left and right side of embryos, only the ISAs and sprouts closest to the objective were analyzed.

To quantify embryos with somatic mosaicism in Fig. 3g–h, embryos were presorted for GFP expression in endothelial cells between 44–50hpf. Only the mosaic segments (area between two adjacent somite boundaries) which contained patches of *kdrl:GFP* or *kdrl:DNBmprI-GFP* expressing endothelial cells were quantified. The number of endothelial branches per segment was counted, and an average was calculated (Fig. 3g). To calculate the percentage of mosaic segments that form a CVP (Fig. 3h), each segment that contained a CVP with a fused ventral vein was given a value of 1, and segments that lacked a fused ventral vein in the CVP were given a value of 0. These values were then used to calculate the percentage of segments with a CVP.

In all cases, embryos with gross morphological defects were presorted and excluded from analysis prior to quantification.

Morpholino injections and small molecule treatment

Microinjections of MOs were performed as previously described[35]. Briefly, embryos were injected at the single cell stage with 4–12ng of control MO (Gene Tools), 12ng of *bmpr2a* splicing MO #1, 12ng of *bmpr2a* splicing MO #2, 8ng of *bmpr2b* splicing MO #1, 12ng of *bmpr2b* splicing MO #2 and a combination of 2ng of *kdrl*, and 2ng of *kdr* MO (Gene Tools). Embryos were co-injected with 2ng of p53 MO (Gene Tools) and embryos with gross morphological defects were presorted and excluded from quantification. The sequences for the MOs used in this study are: *bmpr2a #1*: 5′-AGAGAAACGTATTTGCATACCTTGC-3′; *bmpr2a* #2: 5'-TCATTACGGAAACATACCTCTTAGC-3';*bmpr2b #1*: 5′AGTTGATTCTGACCTTGTTTGACCA-3′; *bmpr2b* #2: 5'-CGGCTTCATCTTGTTCTGACCTCAC-3'; *kdrl*: 5′-CACAAAAAGCGCACACTTACCATGT-3′[5]; and *kdr*: 5′-GTTTTCTTGATCTCACCTGAACCCT -3′[5].

Embryos were treated with chemical inhibitors at 26hpf. The final concentration of small molecule inhibitors was 60μM of SL327, 200μM of SB203580, 40μM of dorsomorphin, 10μM of DMHI, and 5μM of DMHI in 2% DMSO.

Live Imaging and 3-D Image Processing

Embryos were dechorionated, and embedded in 1% agarose (containing egg water with tricaine) in the center of a glass bottom petri dish (MatTek). Once agarose solidified, egg water with tricaine was added. Embryos were imaged using a Zeiss 510 Meta confocal microscope.

Zeiss LSM software was used to generate monochrome projections and 3-D color projections from confocal Z-stacks. The color bar on the 3-D color projections represents the z-axis location of objects with red representing the most proximal (closest to viewer) and blue representing the most distal blood vessels (farthest from viewer).

Real-Time PCR

Quantitative RT-PCR for zebrafish *id2a, vegfa, vegfc, dll4,* and *flt4* was performed using the TaqMan gene expression assay (Applied Biosystems). Wild-type and $Tg(hsp70l:bmp2b)^{+/-}$ fish were incrossed and heat-shocked as previously described. Total RNA was extracted from ~50 embryos 2 hours post heat-shock and 5 hours post heat-shock. *gapdh* was used as an endogenous control to normalize expression levels. The expression of *id2a, vegfa, vegfc, dll4,* and *flt4* were displayed as a ratio of *bmp2b*-induced to wild-type.

Generating Transgenic Constructs

The *vegfa* gene was amplified from cDNA of 32hpf embryos. The PCR product was ligated into the pCR8 vector (Invitrogen). The *vegfa* gene was sequenced and found to be the $vegfaa_{121}$ splicing isoform. The gateway tol2 kit[36] was used to create the $hsp70l:vegfaa_{121}$ construct, which was injected with transposase RNA into 1-cell embryos to create stable $Tg(hsp70l:vegfaa_{121};cmlc2:EGFP)$ transgenic lines.

The dominant negative form of Bmp receptor type I (*DNbmprI-GFP*) gene was amplified from the cDNA of *Tg(hsp70l:DNbmprI-GFP)* embryos[32], and ligated into the pCR8 vector (Invitrogen). The gateway tol2 kit was used to generate the *kdrl:DNbmprI-GFP* construct. The resulting construct was injected with transposase RNA into 1-cell embryos, which generated patches of endothelial cells that over-express the *DNbmprI-GFP* fusion protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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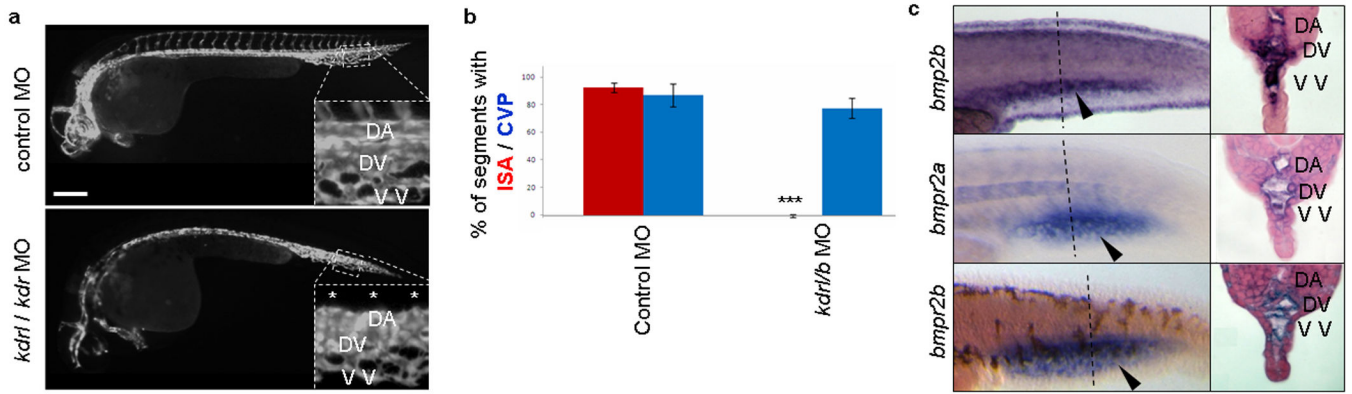
Figure 1. The AV forms angiogenic sprouts despite loss of Vegf receptor activity, and expresses Bmp pathway components
(a) Epiflourescent images of 34hpf *Tg(kdrl:GFP)* control and *kdrl/kdr* MO injected embryos; insets show higher magnification of the CVP region. Asterisks denote the lack of intersegmental arteries in *kdrl/kdr* MO injected embryos. Scale bar, 250μm. (b) The percentage of segments that contain an ISA (red bars) or a CVP (blue bars) was quantified in control (n=9) and *kdrl/kdr* (n=10) MO injected embryos. *kdrl/kdr* MOs completely blocked the formation of arteries but not veins. Error bars represent mean ± SEM. ***P<0.001 versus control, Student's *t* test. (c) Expression pattern of *bmp2b*, *bmpr2a*, and *bmpr2b* in the developing CVP region (black arrowheads) at 32hpf, as detected by *in situ* hybridization. Cross sections from different 32hpf embryos were taken at the area marked by dashed line. Abbreviations: DA, DA; VV, ventral vein; DV, dorsal vein.
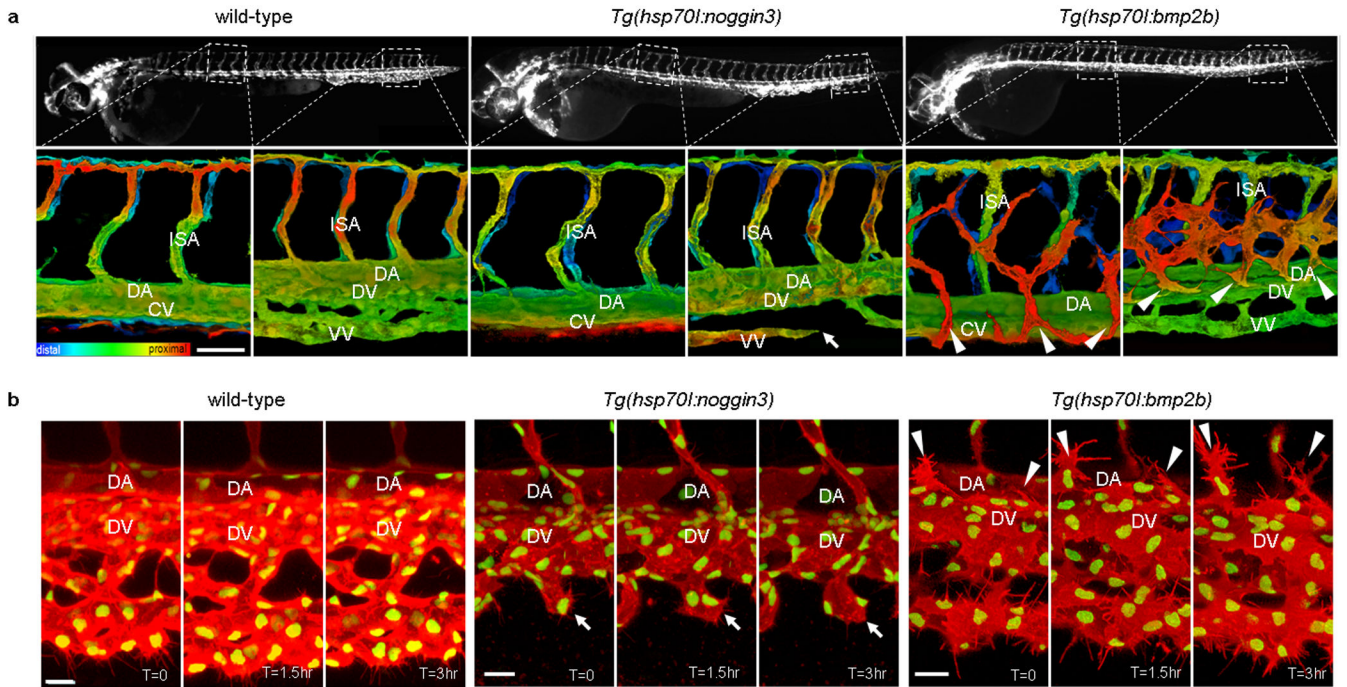
Figure 2. Bmp signaling is necessary and sufficient for sprouting from the AV
(a) Blood vessels in wild-type, *Tg(hsp70:noggin3)*, and *Tg(hsp70:bmp2b)* embryos in the *Tg(kdrl:GFP)* transgenic background. The entire vascular network of 42hpf embryos was analyzed using epiflourescent images; dashed boxes represent the trunk and tail areas analyzed below. Z-stacks from the trunk and tail regions were used to make 3-D color projections, where red represents the most proximal (closest to viewer) and blue represents the most distal (farthest from viewer) blood vessels (epiflourescent images and 3-D color projections were taken from different embryos). Scale bar, 50μm. (b)Time lapse imaging of *Tg(fli1:nGFP);Tg(kdrl:ras-mCherry)* embryos starting at 32hpf. Arrows in panel a and b show sprouts from the AV that fail to make connections in *Tg(hsp70:noggin3)* embryos. Arrowheads in panel a and b point to ectopic sprouts that branch from the AV in *Tg(hsp70:bmp2b)* embryos. Scale bar, 20μm. Abbreviations: DA, DA; VV, ventral vein; DV, dorsal vein; NC, notocord; NT, neural tube; ISA, intersegmental artery.
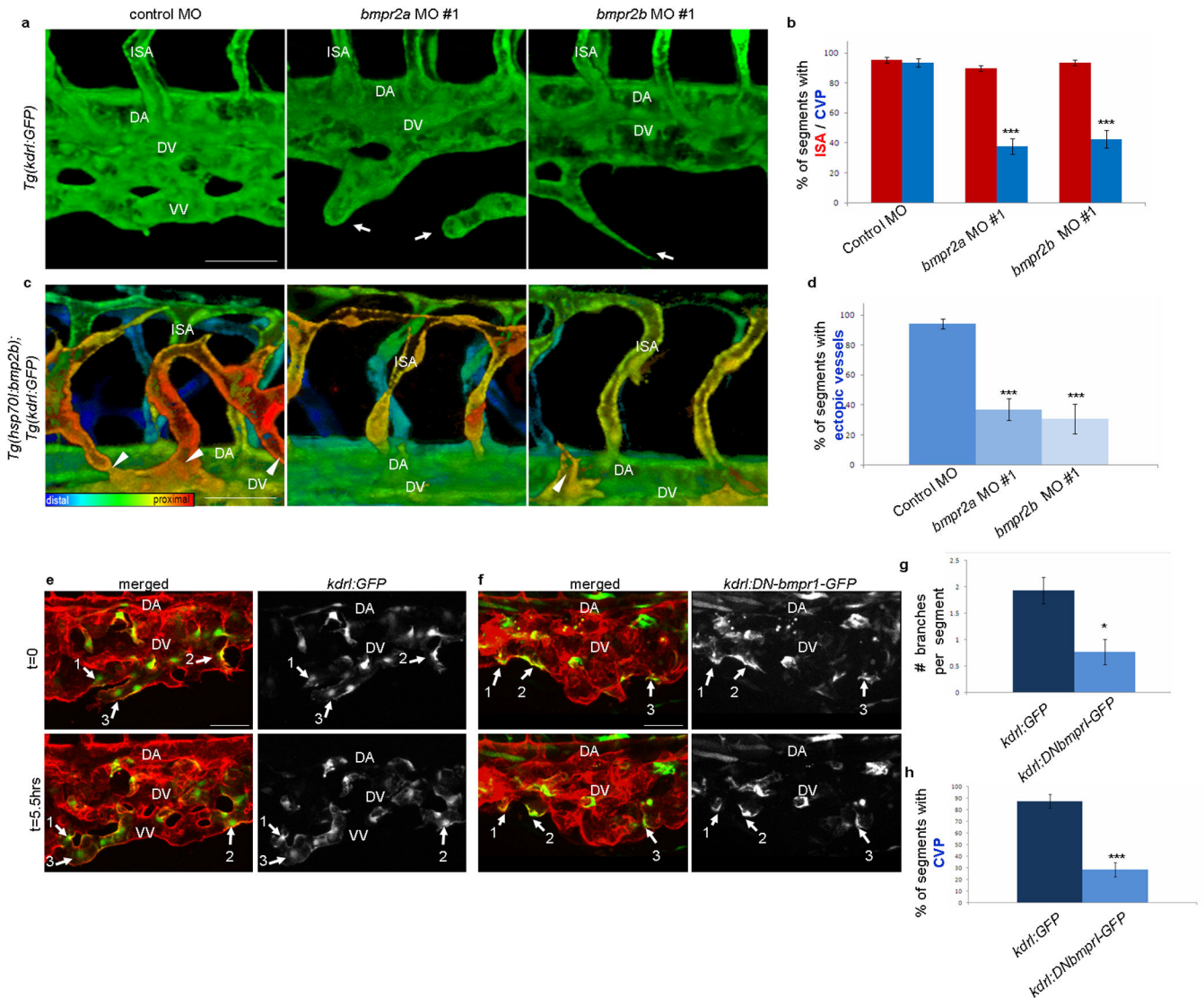
Figure 3. Angiogenesis from the AV requires *bmpr2a* and *bmpr2b* and involves endothelial cell autonomous activation of Bmp signaling

(a) Confocal monochrome projections of *Tg(kdrl:GFP)* embryos injected with a standard control, *bmpr2a*, or *bmpr2b* MO. The sprouts from the AV are disrupted with *bmpr2a* and *bmpr2b* MO (arrows). (b) The percentage of segments that contain an ISA (red bars) or a CVP (blue bars) was quantified. Total of eight embryos were used for the quantification in each case. *bmpr2a* or *bmpr2b* MOs blocked the formation of veins but not arteries. (c) Confocal color depth-code projections of *Tg(hsp70l:bmp2b);Tg(kdrl:GFP)* heat-shocked embryos injected with a standard control, *bmpr2a*, or *bmpr2b* MO. The ectopic sprouts (arrowheads) are reduced in both *bmpr2a* and *bmpr2b* morphants. (d) The percentage of segments that contain an ectopic sprout was quantified in control (n=37), *bmpr2a* #1 (n=27), and *bmpr2b* #1 (n=15) MO injected embryos. The number of Bmp-induced ectopic sprouts was significantly reduced in both *bmpr2a* and *bmpr2b* morphants. (e–f) Time-lapse confocal images of *Tg(kdrl:GFP)* (e) and *Tg(kdrl:DNBmpr1-GFP)* (f) mosaic embryos in a *Tg(kdrl:ras-mCherry)* background. Numbered arrows indicate mosaic endothelial cells. (g)

The number of branch points and (h) the percent of segments containing a CVP were quantified in mosaic segment containing *GFP* or *DNBmprI-GFP* cells. Total of 29 segments in 7 embryos for *Tg(kdrl:GFP)* and 34 segments in 11 embryos for *Tg(kdrl:DNBmpr1-GFP)* were used for quantification (See Methods for detailed quantification method). *DNBmprI-GFP*-expressing endothelial cells contain fewer branches (g) and fail to form proper CVP connections (h). Scale bar, 50μm. Error bars represent mean ± SEM. **P<0.01 and ***P<0.001 versus control, Student's *t* test. Abbreviations: DA, DA; ISA, intersegmental artery; VV, ventral vein; DV, dorsal vein.
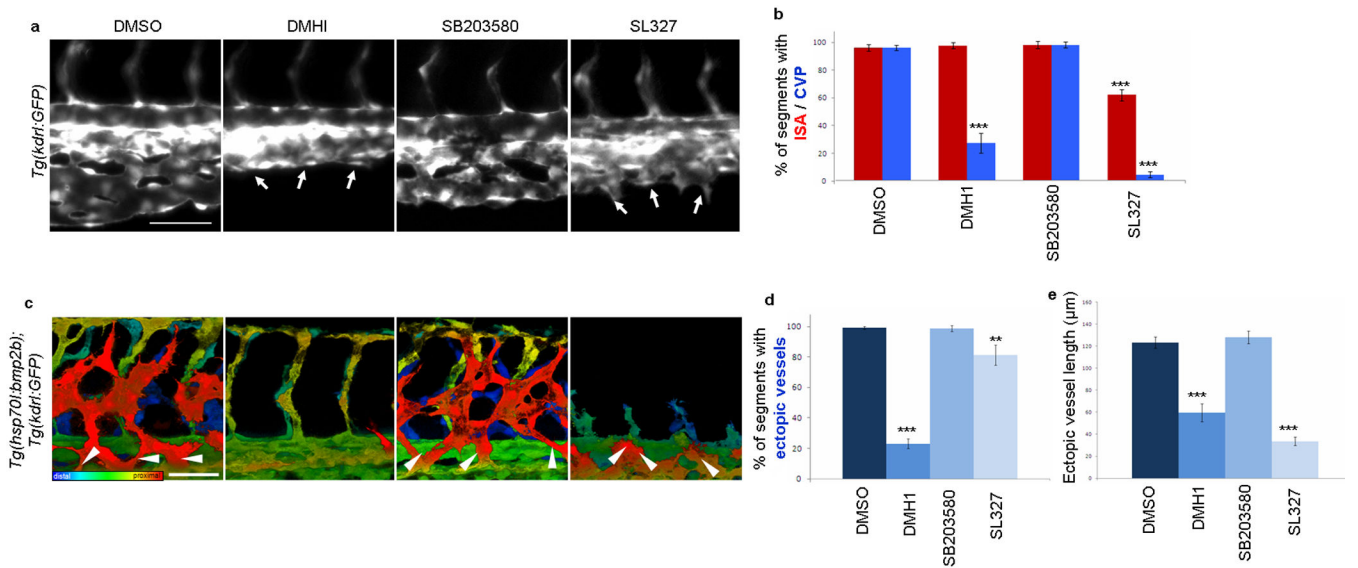
Figure 4. Activation of R-Smad and Erk mediates Bmp-induced angiogenesis
(a) Epiflourescent micrographs of *Tg(kdrl:GFP)* embryos at 38hpf were taken after treatment with DMSO, DMH1 (R-Smad inhibitor), SB203580 (p38 inhibitor), and SL327 (Erk inhibitor). Arrows point to defects in the formation of venous vessels in DMH1- or SL327-treated embryos. (b) The percentage of segments that contain an ISA (red bars) or a CVP (blue bars) was quantified in DMSO (n=14), DMH1 (n=13), SB203580 (n=8), or SL327 (n=10) treated embryos. (c) Confocal depth-code color projections of *Tg(hsp70l:bmp2b);Tg(kdrl:GFP)* embryos at 46hpf were taken after treatment with small molecule inhibitors. Addition of DMH1 or SL327 to *bmp2b* over-expressing embryos inhibited Bmp-induced ectopic sprouts. Arrowheads point to ectopic sprouts from the AV. (d) The percentage of segments that contain an ectopic vessel was quantified in DMSO (n=11), DMH1 (n=13), SB203580 (n=4), or SL327 (n=6) treated embryos. (e) The average ectopic vessel length was quantified in DMSO (n=15), DMH1 (n=14), SB203580 (n=16), or SL327 (n= 22) treated embryos. Inhibition of either R-Smad or Erk activation significantly reduced the formation ectopic vessels and the average length of ectopic vessels. Error bars represent mean ± SEM. **P<0.01 and ***P<0.001 versus control, Student's *t* test.
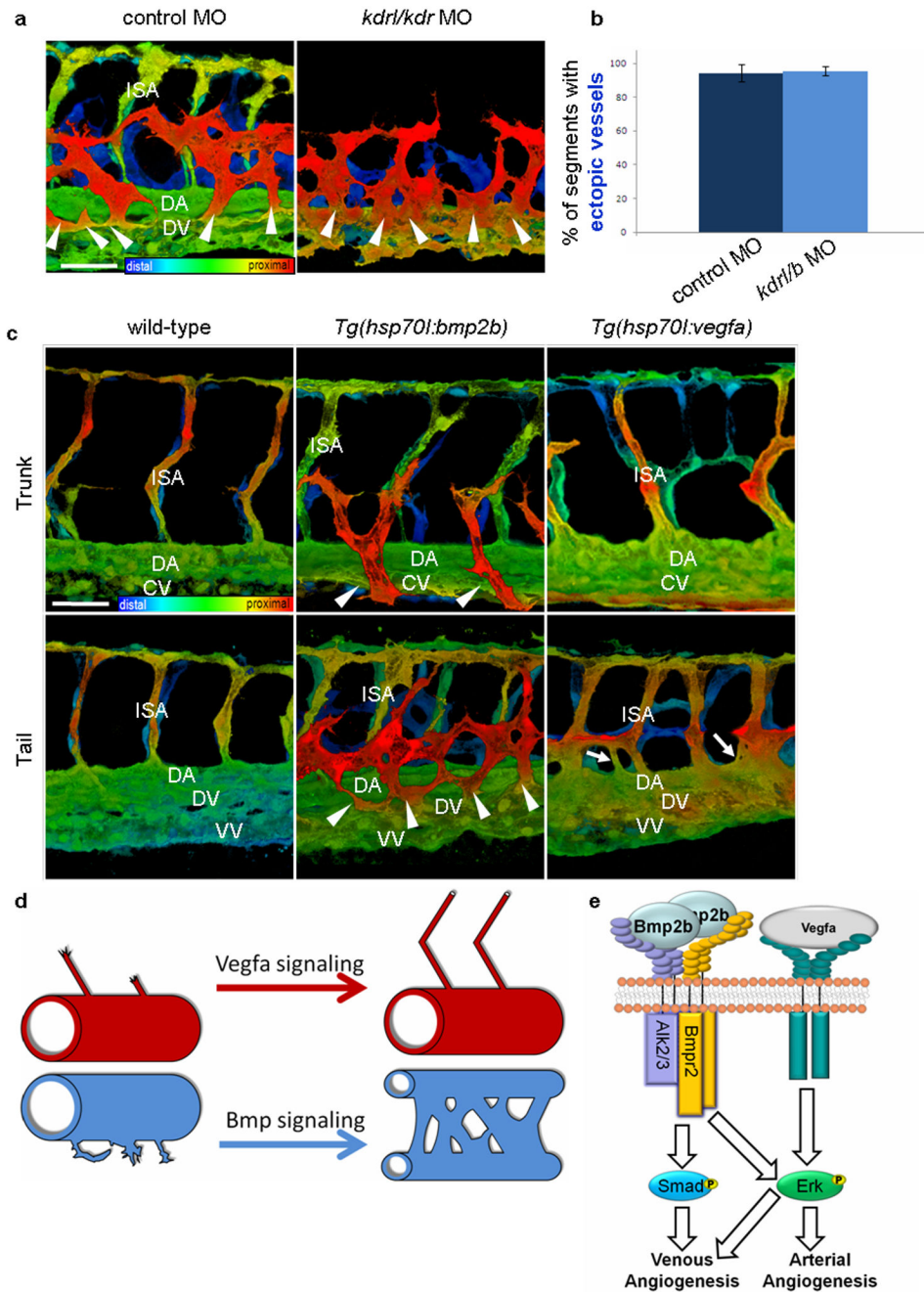
Figure 5. Bmp signaling regulates AV angiogenesis independent of Vegf receptor activity
(a) Control and *kdrl/kdr* MOs were injected into *Tg(hsp70:bmp2b); Tg(kdrl:GFP)* heat-shocked embryos and shown as 3D color projections. The number of Bmp-induced ectopic sprouts (arrowheads) was not affected by the loss of Kdrl/Kdr activity. Scale bar, 50μm. (b) The percentage of segments that contain ectopic vessels was quantified (n=3 for control, and 6 for *kdrl/kdr* MO). There was no statistically significant difference between control and *kdrl/kdr* MOs injected embryos. Error bars represent mean ± SEM. (c) 3-D color projections were taken from the trunk and tail region of 42hpf heat-shocked embryos. Over-expression

of *bmp2b* induced ectopic sprouts in venous endothelial cells (arrowheads), while over-expression of *vegfa* stimulated ectopic sprouts in arterial endothelial cells in the trunk (arrows). (d) In this model, Bmp signaling is the dominant regulator of AV angiogenesis, while Vegf-A is the main regulator of angiogenesis from the DA. (e) In venous endothelial cells, Bmp2b ligand binds to a Bmpr2a and/or Bmpr2b and Alk2/Alk3 hetero-tetrameric complex, which phosphorylates R-Smad and Erk to promote angiogenesis, while arterial cells utilize the classical Vegf-A signaling cascade to induce angiogenesis. Scale bar, 50μm. Abbreviations: DA, DA; VV, ventral vein; DV, dorsal vein; ISA, intersegmental artery.